Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science
Department of Chemistry


# Study the Effect of Aromatase Inhibition in Postmenopausal Breast Cancer Patients and Its Cytotoxic Effect *In-Vitro*

## A Thesis

Submitted to the college of science / Al-Nahrain University as partial fulfillment of the requirements for the Degree of Master of Science in chemistry

**By**

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March 2016 Jamadi alakhar1437

بسم الله الرحمن الرحيم

يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ {11}

صدق الله العظيم

سورة المجادلة

# Dedication

*To the person who was always supported me to reach to the best...*

*My Dearest*

*Father*

*To the fountain of patience and optimism and hope …*

*My lovely*

*Mother*

*To persons who always give me the hope and reason to go on in my live …*

*Brothers and sister*

*My refuge when I anger and gladness…*

*My Best friend*

*zainab*

*MAYS*

# *Supervisor Certification*

I certify that this thesis was prepared under my supervision at Chemistry Department, College of Science, Al-Nahrain University, in partial fulfillment of the requirements for the degree of Master of Science in Chemistry.

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# Committee certification

We, the examining committee certify that we have read this thesis entitled "**Study the Effect of Aromatase Inhibition in Postmenopausal Breast Cancer Patients and Its Cytotoxic Effect In-Vitro"** and examined the student "**Mays Waad Abd-Allateef**" in its contents and that in our opinion; it is accepted for the Degree of Master of Science, in biochemistry.

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I, hereby certify upon the decision of the examining committee.

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# Acknowledgement

*Thanking **GOD** for giving me the power and health to complete this work.*

*I would like to express my gratitude to my supervisor **Dr. Firas A. Hassan**, for their valuable guidance and encouragement in supervising the present work.*

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*Special thanks to patients and healthy people to help me by giving blood samples.*

*My special thanks to who standing by my side all the time in study and life, my best friend **Zainab Salih** , thanks for your support.*

***Mays Waad***

# Summary

***Background*:**

Hormone therapy is the first targeted therapy also called estrogen suppression therapy often used as an adjuvant therapy to reduce the risk of cancer back after surgery or cancer that has spread. Aromatase inhibitors such as (anastrozole) are a common type of the hormone therapy; it interferes with the body's ability to produce estrogen from androgen by suppressing aromatase enzyme in post-menopausal women with positive estrogen receptor breast cancer.

Present study was planned to assess the effect of anastrozole on aromatization mechanism According to aromatase enzyme activity and estradiol level. As well, study the cytotoxic effect of anastrozole on three types of cell line (breast, liver and prostate) and survey the apoptosis mechanism for MCF-7.

***Subjects and methods:***

This study includes (80) post-menopausal women, their age ranged from (45-75) years. Twenty subjects were apparently healthy chosen as control group (C group) and sixty patients with breast cancer (P group), were divided into two groups according to taken the treatment: twenty patients without treatment (newly diagnosed) (P1 group), forty patients with treated by (1 mg per day) anastrozole (P2 group). The levels of all parameters were quantitatively determined in patients and control subjects by ELISA device. The Second part of this study was determination the cytotoxic effect of anastrozole on three types of cell line and investigation the mechanism by which the affected of anastrozole in living cells toward apoptosis by High Content Screening (HCS) assay.

***Results***:

Part I :

- A significant increase in the mean of BMI was observed in the (P1) group and (P2) group in comparison with that of the group (C) (32.56 and 30.98 vs. 25.44 $Kg/m^2$).
- The mean of serum Estradiol level was showed a significant increase of group (P1) and group (P2) in comparison with that group (C) (18.10 and 13.09 vs. 12.29) while the results shown a significant decrease in the group (P2) when comparison with a group (P1)
- The mean of serum aromatase level was showed a significant increase of group (P1) and group (P2) in comparison with that group (C) (746.24 and 452.34 vs. 347.97). while there was significant decrease in group (P2) when comparison with a group (P1)
- Serum aromatase level of the group (P1) revealed a significant negative correlation with waist, height and WHR while there was a significant positive correlation between estradiol and BMI and weight with the group (P1) and (P2).
- Personal correlation of serum estradiol level of the group (P1) revealed a significant negative correlation with waist, height and WHR while shown significant positive correlation with aromatase and BMI and weight with (P1) and (P2) groups.

Part II :

- Cytotoxic effect of anastrozole in three types of cell line was showed that 400µg/ml was the most significant cytotoxic toward all cell lines treated for 24 hours, but the MCF-7 cells were more sensitive to anastrozole than other cell lines in present study.

- The effect of the anastrozole on the mechanism of apoptosis was detected a significant increase with increasing the concentration (dose depended) for cell membrane permeability, cytochrome c and nuclear intensity at (200µg/ml of anastrozole) when comparison with (20µg/ml) of doxorubicin as a standard.
- The effect of the anastrozole on the mechanism of apoptosis was detected a significant decrease with increasing the concentration (dose depended) for cell viability and Mitochondrial Membrane Permeability at (200µg/ml) when comparison with 20 µg/ml of doxorubicin as a positive control.

***Conclusions:***

- Appear a positive and strong correlation between aromatase enzyme and estradiol, moreover revealed decrease significant in same parameters on patients with treatment, which that confirms the effectiveness of anastrozole and hypothesis of aromatization.
- Anastrozole was possessing cytotoxic effect against breast cancer cells (MCF-7), liver hepatocellular cancer cells (HepG2) and prostate cancer cells (PC-3), determined in-vitro by MTT assay.
- The HCS technique for the anastrozole showed toxic effect toward MCF7 cell line at (200µg/ml) anastrozole concentration in a dose-dependent manner with increasing in cell membrane permeability, cytochrome c, nuclear intensity, changing in mitochondrial membrane potential and decreasing in cell viability level.
- Anastrozole has two effects on breast cancer, inhibition of aromatase enzyme that lead to inhibit aromatization mechanism and toxic effect.

# Abbreviations

| | |
|---|---|
| ADME | Absorption,distribution, metabolism, and excretion |
| AIs | Aromatase inhibitors |
| ANOVA | Analysis of variance |
| BCT | Breast-conserving therapy |
| BMI | Body mass index |
| Cyt | Cytochrome |
| E1 | Estron |
| E2 | Estradiol |
| E3 | Estriol |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Estrogen receptor |
| HCS | High content screening |
| HepG2 | Liver hepatocellular cancer cell |
| HER2 | Human epidermal growth factor receptor 2 |
| LSD | Least significant difference |
| MCF7 | Breast cancer cell line |
| MPT | Mitochondrial permeability transition |
| MRI | Magnetic Resonance Imaging |
| MTT | (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (tetrazole) |
| N.S. | Non-significant |
| P-value | Probability value |
| PC3 | Prostate cancer cell |

| | |
|---|---|
| PR | Progesterone receptor |
| r- value | Person correlation coefficient |
| RFA | Radiofrequency ablation |
| RT | Radiation therapy |
| SN | Sentinel node |
| TNF-R | Tumor necrosis factor receptors |
| TNM | Tumor, node and metastasis |
| WHO | World health organization |
| WHR | Waist-to-height ratio |
| WHtR | Waist-to-hip ratio |

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# CHAPTER ONE

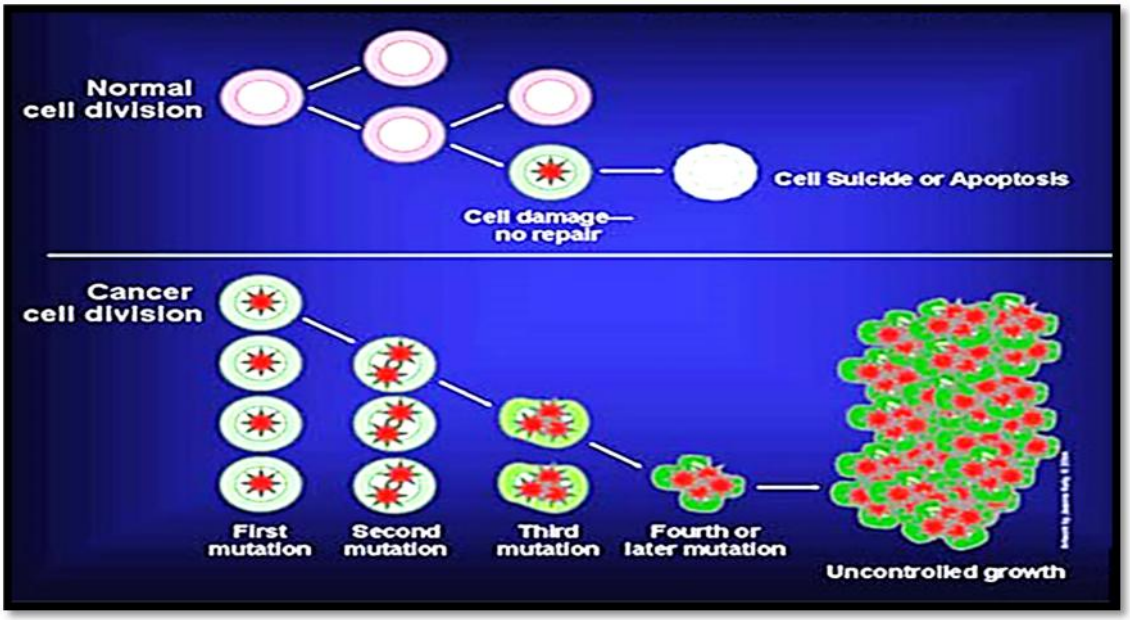
# INTRODUCTION AND LITERATURE REVIEW

# 1. Introduction

## 1.1 Cancers

Cancer is the uncontrolled growth of abnormal cells in the body [1] which result from a series of molecular events that fundamentally alter the normal properties of cells. In cancer cells, the normal control systems that prevent cell overgrowth and the invasion of other tissues are disabled [2]. These altered cells divide and grow, display uncontrolled growth, invasion and sometimes metastasis, according to these, altered cells divide and grow in the presence of signals that normally inhibit cell growth. The abnormalities in cancer cells usually result from mutations in protein-encoding genes that regulate cell division [3].

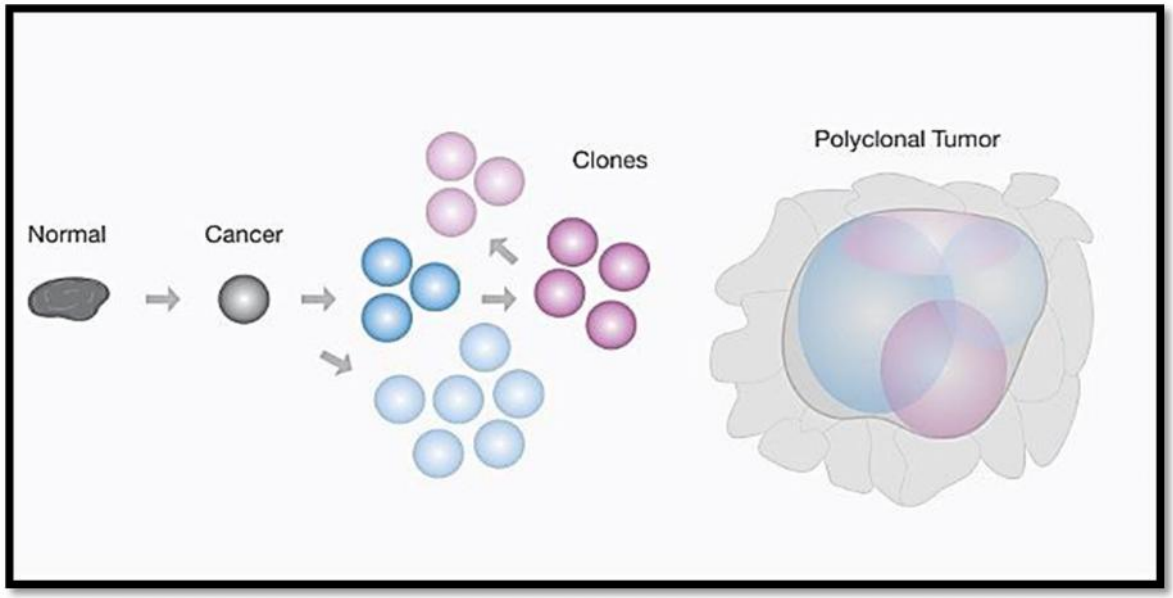
Over time, more genes become mutated; this is often because the genes that make the proteins that normally repair DNA damage are themselves not functioning normally because they are also mutated. Consequently, mutations begin to increase in the cell, causing further abnormalities in that cell and the daughter cells (scheme 1-1) [4]. Some of these mutated cells die, but other alterations may give the abnormal cell a selective advantage that allows it to multiply much more rapidly than the normal cells, this enhanced growth describes most cancer cells, which have gained functions repressed in the normal, healthy cells [5].




Scheme (1-1): comparison between normal and malignant cells[4].

If these cells remain in their original location, they are considering benign; while if they become invasive, they are considered malignant. Cancer cells in malignant tumors can often metastasize, spread of cancer from one organ or part to another not directly connected with it [6].

The tumor itself may be heterogeneous. During cancer progression, new mutations may occur in individual cells and these newly mutated cells can go onto proliferate and form clones. As a result, late-stage cancers often consist of polyclonal tumors, where every clone has a unique set of mutations and unique drug responses as shown in the scheme (1-2) [7, 8].

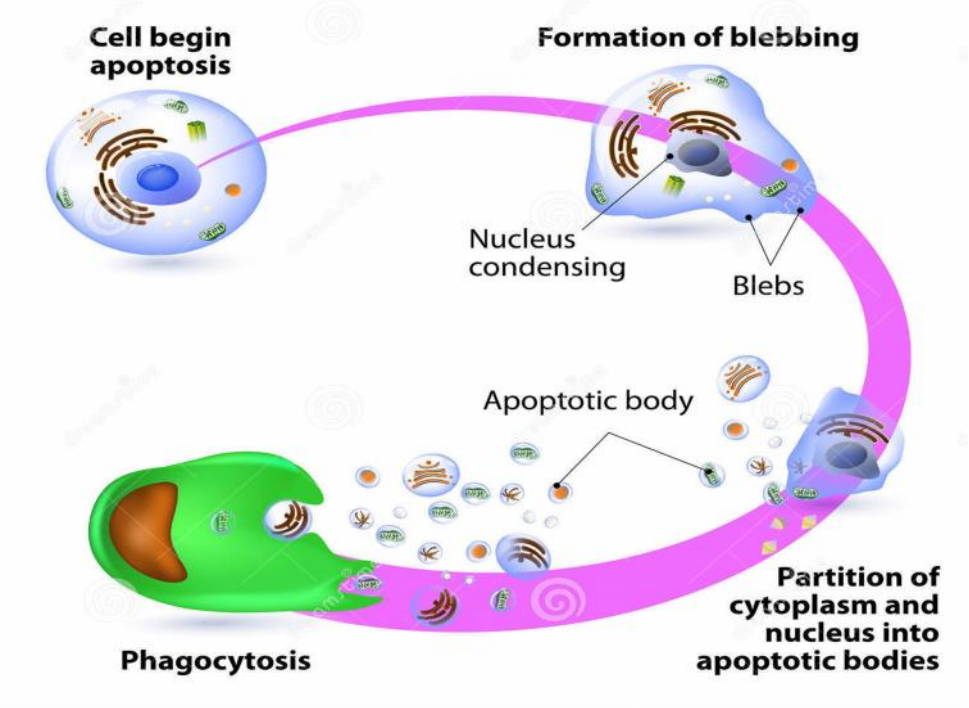



Scheme (1-2): heterogeneous polyclonal tumor in which different clones[8].

## 1.1.1. Apoptosis

Apoptosis is a term coined by Kerr, Wyllie and Currie in 1972, characterized by an ordered series of physical and biochemical reactions that are controlled by a variety of genes such as $P_{53}$ and Bcl-2 [9].

Apoptosis is a one kind of the process of programmed cell death that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blubbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay [10]. Also, it is a highly regulated and controlled process that confers advantages during an organism's lifecycle. Apoptosis produces cell fragments called apoptotic bodies that phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out onto surrounding cells and cause damage, (scheme 1-3)[11].




Scheme (1-3): cellular change during apoptosis[11].

Apoptosis and cancer are connected in two important ways. First, tumors cannot grow if the normal safety system within the body detects them and triggers their self-destruction, so cancers often block the mechanisms by which the body marks uncontrolled tissue growth for apoptosis. Secondly, cancer cells inactivate the genes that encode the necessary proteins to destroy malignant cells [12].

## 1.1.2 Molecular mechanism of apoptosis

Apoptosis may be triggered by two major mechanisms: the intrinsic (mitochondrial) and extrinsic (death receptor) mediated pathway [13]. The mitochondria pathway of cell death can be activated by a variety of receptor-independent stimuli such as radiation, free radicals, viral infections and serum/growth factor withdrawal [14]. Initially, it was

demonstrated that these triggers invariably result in changes in the inner mitochondrial membrane permeability due to the opening of the mitochondrial permeability transition (MPT) pore. The major consequences of this change of permeability are the loss of the mitochondrial transmembrane potential, the release of pro-apoptotic proteins and the arrest of the bioenergetic function of the organelle. The proteins that are released can be broadly classified into two categories. The first category, certain proteins (e.g., BCL-2, BCL-xL) act to preserve mitochondrial integrity by preventing loss of mitochondrial membrane potential and/or release of pro-apoptotic proteins such as cytochrome C into the cytosol [15]. Second category, proteins such as the inhibitors of apoptosis proteins (e.g., XIAP) or FLIP block the activation of caspases, particularly those involved in engagement of the receptor-related [16].

Death receptors are cell surface receptors that transmit apoptosis signals initiated by their specific “death ligands” [17]. The cell surface death receptors belong to the superfamily of tumor necrosis factor receptors (TNF-R) and are activated by TNF family ligands. This pathway comprises several protein members, including the death receptors, the membrane-bound FasL, the Fas complexes, the Fas-associated death domain and caspases 8 and 10, which ultimately activate the rest of the downstream caspases, leading to apoptosis [18].

## 1.2 Breast cancer

Breast cancer is common cancer among women in the world and the single leading cause of death in women aged 40 to 50. It has been estimated that 1,200,000 new cases occur every year worldwide [19] and represents 11 % among all cancer types globally [20]. In the United States about 178,480 women affected by breast cancer [21]. In Iraq, where the population was exposed to high levels of depleted uranium following the first and second Gulf Wars, breast cancer is the most common cancer type in females. Over the last years, there has been a three-fold increase in the incidence of breast cancer [22]

Breast cancer begins in the breast tissue that is made up of glands for milk production, called lobules, and the ducts that connect the lobules to the nipple. The remainder of the breast is made up of fatty, connective, and lymphatic tissues [23]. Also, it is considered a highly heterogeneous group of cancers arising from different cell types and each having its own clinical implications [24]. Currently, all breast cancers are tested for expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal growth factor Receptor 2 (HER2/neu) proteins. ER and PR tests are usually done by immunohistochemistry whereas HER2/neu is accessed by FISH. These proteins profiling of tumors helps to predict the eventual prognosis and can assist in the determination of the most appropriate treatment for the individual [25].

ERs are over-expressed in around 70% of breast cancer cases, and are referred to as "*ER-positive*" tumors [26]. Binding of estrogen to ER stimulates proliferation of mammary cells, with the resulting increase in cell division and DNA replication and increases mutation rate. This causes disruption of the cell cycle, apoptosis and DNA repair processes

eventually leading to tumor formation. Additionally, estrogen metabolism leads to the production of genotoxic by-products that could directly damage DNA, resulting in point mutations [27].

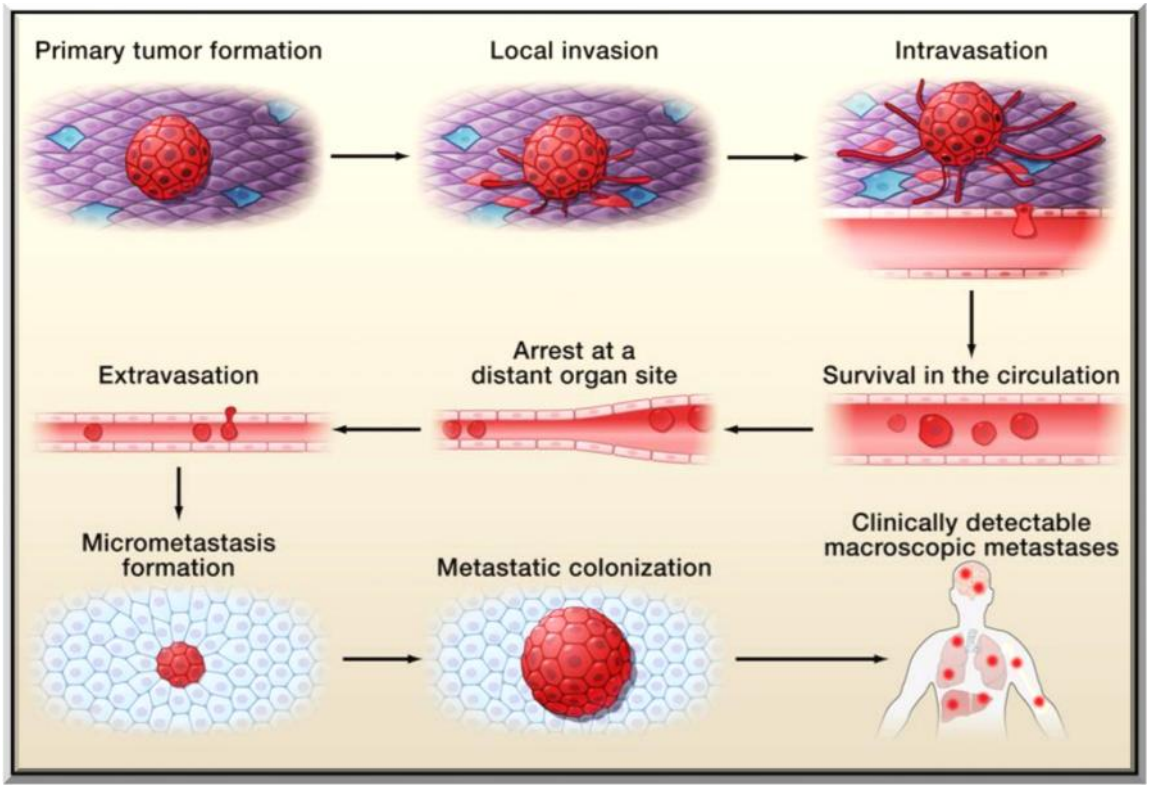
About 65% of ER-positive breast cancers are also PR-positive and about 5% of breast cancers are ER-negative and PR-positive. If cells have receptors for both hormones or receptors for one of the two hormones, the cancer is considered hormone-receptor positive [28].

HER2/neu is a protein giving higher aggressiveness in breast cancers [29]. It is a member of the ErbB protein family, more commonly known as the epidermal growth factor receptor family. Approximately 30% of breast and ovarian cancers have an amplification of the HER2/neu gene or over-expression of its protein product [30].

## 1.2.1 Breast cancer anatomy

Breast cancer is the malignant tumor caused by uncontrolled growth of cells in the breast. It is the most common cancer among women [31]. Most breast cancers begin in the milk ducts. These ducts connect the milk-producing glands (called lobules) to the nipple [32]. Some breast cancer begins in the lobules themselves, and the rest begins in other tissues. Once the cells have broken through the wall of a duct or lobule, the cancer is called invasive. If the cells then travel into the lymphatic vessels of the breast, they may be carried to other parts of the body so that cancer spreads, scheme (1-4). This process is called metastasis. Metastasis is a complex process in which cancer cells break away from the primary tumor and circulate through the bloodstream or lymphatic system to other sites in the body. At new sites, the cells continue to

multiply and eventually form additional tumors comprised of cells that reflect the tissue of origin [33].




Scheme (1-4): Showed Invasive and Metastasis of Breast Cancer [34].

However, not all breast tumors are considered to be cancer because certain type of large cell collection cannot spread or threaten a person's life; this type of tumor is called a benign tumor [35], as shown in figure (1-1). After a diagnosis of benign cancer, there is currently a 5-year survival rate of 97% [36].

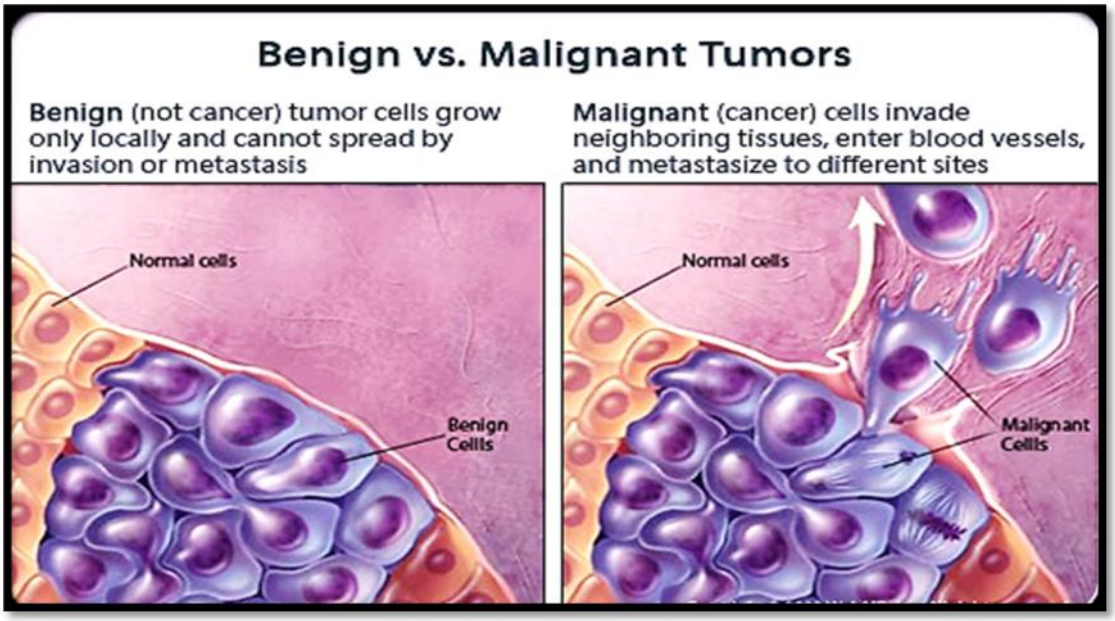



Figure (1-1): compare between benign and malignant tumors[36].

## 1.2.2 Staging of breast cancer

Cancer staging systems describe how far cancer has spread anatomically and attempt to put patients with similar prognosis and treatment in the same staging group. The staging system normally used in breast cancer is called TNM [37], which stands for ‘tumor, node, and metastasis. So TNM staging takes into account the size of the tumor, whether the lymph nodes are affected, and whether cancer has spread to other parts of the body (metastasis) [38]. A simplified version of the TNM system is displayed in Tables (1-1).

**Table** (1-1): Subdivisions of the TNM Staging System [39].

| **Tumours** | **T0/Tis** | **T1** | **T2** | **T3** | **T4** |
|---|---|---|---|---|---|
| **Tumour Size** | **T0**: No primary tumour. **Tis**: Tumour only in breast ducts or lobules. | 0-2 cm | 2-5 cm | >5 cm | Tumor of any size with extension to chest wall/skin or ulceration ****inflammatory breast cancer** is staged as T4. |
| **Nodes** | **N0** | **N1** | **N1mi** | **N2** | **N3** |
| | No lymph node metastases. | Cancer cells present in 1-3 axillary lymph nodes. | Lymph node tumor > 2 mm. | Cancer cells present in 4-9 axillary lymph nodes. | Cancer cells in infra or supraclavicular lymph nodes, or in >10 axillary lymph nodes. |
| **Metastasis** | **M0** | **M1** | | | |
| | No evidence of cancer metastasis. | Cancer found in other areas of body. | | | |

## 1.3 Risk factor

Women with a family history of breast cancer should obtain as much information as possible about those relatives, including age at onset and type of cancer. The risk of breast cancer development related to family history increases with the number of affected relatives, specific lineage and age at diagnosis. The younger age at diagnosis, the more likely that a genetic component may be involved. About 5-10% of breast cancer is thought to be linked to changes (mutations) in certain genes [40, 41]. However, most cases of breast cancer occur “by chance”. The causes

are still unknown, but there is probably a combination of factors including lifestyle factors, environmental factors and hormone factors.

A list of several risk factors for breast cancer is shown below [42].

- Age
- Family history
- Body mass index >35
- Cancer in the other breast
- Exposure to ionizing radiation
- Alcohol consumption (excessive intake)
- Diet with high intake of saturated fat
- Gen factors
- Menstrual and reproductive factors
- Hormone replacement therapy

## 1.4 Tumor Grading

Tumor grade refers to a measure of how abnormal cells from your tumor appear under the microscope. This can refer to the appearance of the cells or to the percentage that appear to be dividing. The higher the grade, the more aggressive and fast growing the cancer. Tumors are typically classified from least to most aggressive as grade I through IV [43].

The grade is much more important for some kinds of cancers than for others. For most kinds, it is a somewhat secondary factor, but for a few kinds of cancers, notably certain breast cancer, brain tumors, prostate cancer, and lymphomas, it is extremely important. Again your doctor will know how your tumor was graded and how important it is to your type of

cancer. The grading will also be found on the pathology report from your biopsy or surgery. For information on understanding pathology reports [44].

## 1.5 Diagnosis

Doctors use many tests to diagnose cancer and find out if the cancer has spread or metastasized to other parts of the body beyond the breast and the lymph nodes under the arm. Some tests may also help the doctor decide which treatments may be the most effective. For most types of cancer, a biopsy is the only way to make a definitive diagnosis of cancer. A biopsy is the removal of a small amount of tissue or cells for examination under a microscope. This list describes options for diagnosing this type of cancer, and not all tests listed will be used for every person [45].

1. Clinical Examination
2. Imaging tests
   - Diagnostic mammography.
   - Ultrasound
   - MRI.
3. Open Biopsy and Frozen Section
4. Biopsy
   - Tumor features
   - ER and PR
   - HER2

# 1.6 Breast cancer Treatment

Breast cancer treatment options vary depending on the stage of cancer, its size, position, whether it has spread to other parts of the body and the physical health of the patient. Current treatments for breast cancer include surgery, radiotherapy, chemotherapy, hormonal and targeted therapies [46]. These therapies may be used alone or in combination depending on the stage of the disease [47].

## 1.6.1 Surgical therapy

Breast cancer surgery has changed dramatically over the past years. With the emergence of breast-conserving therapy (BCT), many women now have the option of preserving a cosmetically acceptable breast without sacrificing survival [48]. Breast conserving therapy is defined as excision of the primary breast tumor with a rim of adjacent normal breast tissue sufficient to achieve negative resection margins, with or without axillary sentinel node (SN) biopsy or dissection, followed by irradiation [49].

## 1.6.2 Minimally invasive procedures

These minimally invasive techniques include endoscopic lumpectomy, vacuum-assisted percutaneous excisional biopsy, and percutaneous thermo ablation with radiofrequency, cryotherapy or laser. Minimally invasive techniques are at the forefront of surgical and interventional innovation and are being evaluated in the setting of clinical trials, as they do not represent current standard of care for breast tumors, benign or malignant [50].

### 1.6.3 Radiofrequency Ablation

Radiofrequency ablation (RFA) is one of the most established forms of local treatment in patients with tumors, which tumors are destroyed *in situ* by thermal coagulation and protein denaturation [51]. High frequency (460 kHz) alternating current flows from un insulated electrode tips into surrounding tissue. As the tissue ions attempt to follow the change in direction of the alternating current, ionic agitation results in frictional heating. The tissue surrounding the electrode, rather than the electrode itself, is the primary source of heat [52]. Although there are several potential mechanisms for cellular injury due to radiofrequency energy, the predominant mechanism is likely thermal injury due to the radiofrequency-induced heating of tissue [53].

### 1.6.4 Cryotherapy

Cryosurgery (also called cryotherapy or cryoablation) is sometimes used to treat early-stage cancer by freezing it. Most doctors do not use cryosurgery as the first treatment for cancer, but it is sometimes an option if cancer has come back after other treatments. Between 1999 and 2007, cryotherapy was carried out on 53 cases with biopsy-proven breast cancer with age range 38-81 years (mean 61). [54]. however it should never be used to treat lesions of uncertain diagnosis [55].

### 1.6.5 Radiotherapy after surgery

Radiotherapy (RT) is one of the primary treatment options in cancer management, effectively saving and prolonging lives. Radiotherapy is widely recognized as one of the safest areas of modern medicine;

however, when errors occur, the consequences for the patient can be significant [56]. Radiotherapy uses high energy x-rays to destroy cancer cells. Some of the normal cells can also be damaged, but they are better at repairing themselves than cancer cells . It may be given before or after surgery. When given before surgery, it may make the tumor smaller. When given after surgery, it will help kill any cancer cells that might be left behind. Sometimes chemotherapy is given at the same time as radiotherapy [57].

### 1.6.6 Adjuvant chemotherapy

Chemotherapy works by stopping or slowing the growth of cancer cells, which grow and divide quickly. But it can also harm healthy cells that divide quickly, such as those that line your mouth and intestines or cause your hair to grow. Damage to healthy cells may cause side effects [58]. The addition of adjuvant chemotherapy to RT results in a decreased incidence of breast recurrence when compared with conservative surgery and RT alone. Early adjuvant systemic chemotherapy in patients at substantial risk of metastases is believed to be important. [59-60].

## 1.6.7 Endocrine therapy

Endocrine therapies also called Hormonal therapy for breast cancer has been used for more than a century [61]. The concept that changing the hormonal balance of the patient with breast cancer could lead to changes in tumor growth and regression of metastatic disease was recognized even before hormones and endocrine agents were available [62].

The history of endocrine therapy for advanced breast cancer started with the seminal discovery by George Beatson (1896) that ovarian ablation may cause tumor regression in premenopausal women. While ovarian estrogen synthesis ceases at the menopause, postmenopausal women still have plasma estrogen levels present at low concentration. Previously believed to occur by adrenal glandular synthesis, it later became clear that the adrenals are contributors of circulating androgens, subsequently converted into estrogens in different body compartments [63].

The mechanisms of action of endocrine therapies are three fold: they may lower the estrogen level in the tumor (oophorectomy, aromatase inhibitors), they may modulate estrogen receptors (SERMS [tamoxifen,toremifene]), or they may modulate the estrogen receptor (ER) with pure antagonist activity, e.g., ER down-regulator (fulvestrant). Although high-dose estrogens, progestin, and androgens have activity against ER+ tumors, the exact mechanism of action is still unclear [64].

Estrogens are a naturally occurring steroid hormone, which functions as the primary female sex hormone. There are three classes of estrogen: estrone (E1), estradiol (E2), and estriol (E3) as shown in figure (1-2), produced primarily by the ovaries; some estrogens are also produced in smaller amounts by other tissues such as the liver, adrenal glands, and the breasts. These secondary sources of estrogens are especially important in postmenopausal women [65], because it will be the source of estrogens when the ovaries cease to produce estrogen [66].

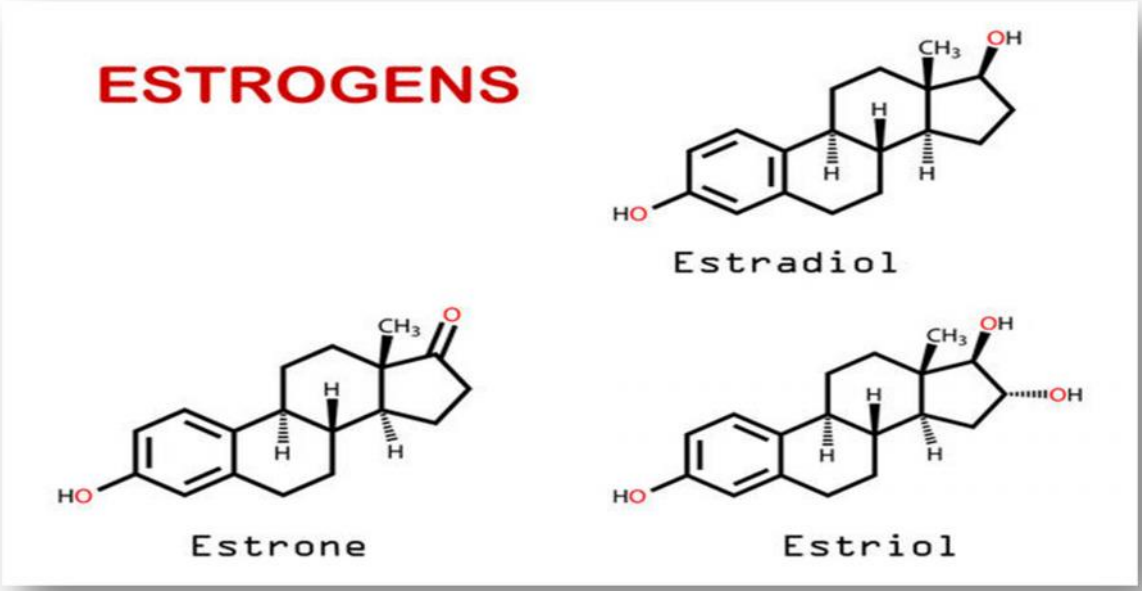



Figure (1-2): classes of estrogen[65].

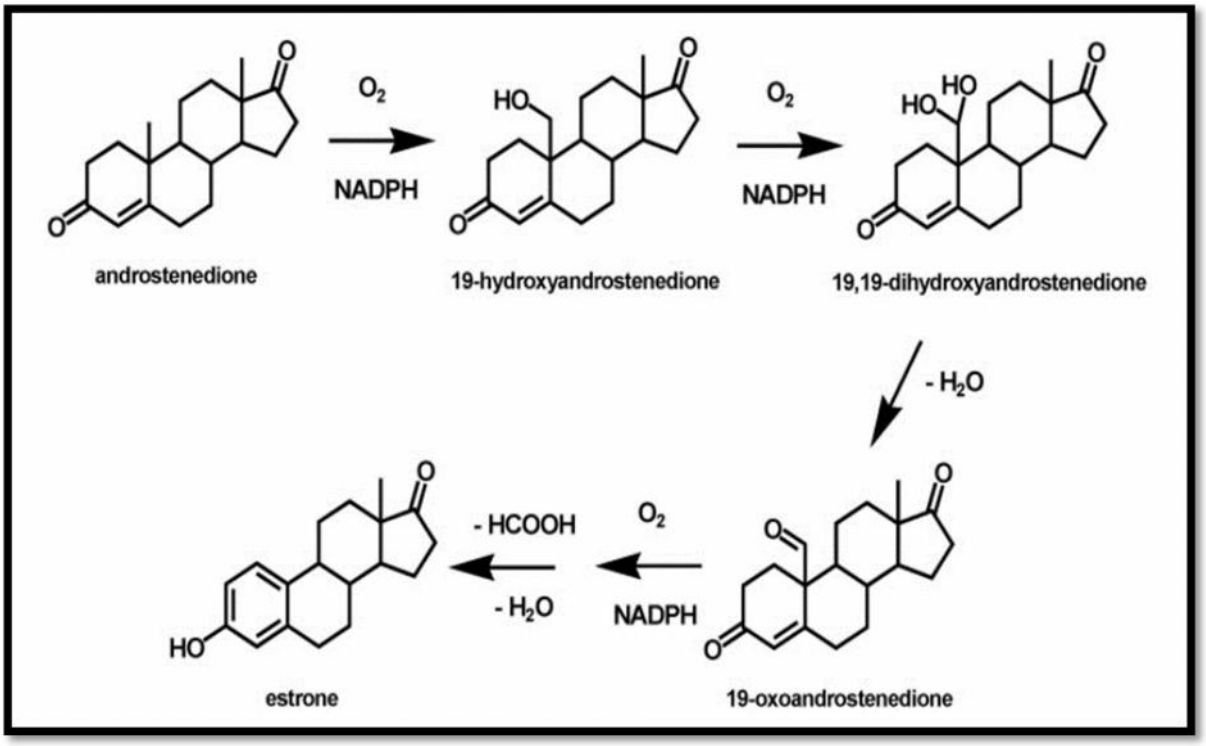
All three types of estrogen are produced from androgens (androstenedione or testosterone), this conversion (aromatization) is catalyzed by aromatase enzyme [67].

## *1.7 Aromatase enzyme*

Aromatase (cytochrome P450), also called estrogen synthetize, is an enzyme responsible for a key step in the biosynthesis of estrogens. Aromatase is a 503-amino acid protein encoded by the CYP19 gene, which is located at 15q21.2 in humans and contains 10 exons [68], it is expressed in several tissues, including subcutaneous fat, liver, muscle, brain, normal breast tissues, and mammary adenocarcinoma (the predominant source of estrogen in postmenopausal women)[69]. It catalyzes the last steps of estrogen biosynthesis from androgens. Specifically transforms androstenedione to estrone (E1) and testosterone to estradiol (E2)(the predominant source of estrogen in postmenopausal

women). This conversion comprises three sequential oxidation steps, each one required 1 mol of $O_2$ and NADPH. In the first step, the androgen is hydroxylated at C-19 to afford the 19 - hydroxy intermediate [70].

The reaction occurs with the retention of configuration, which is characterized by a significant normal kinetic isotope effect. In the second step 19 - hydroxy steroid undergoes second hydroxylation of C-19 to give gem diolic group as shown in scheme (1-5). This on consequent dehydration results in the corresponding aldehyde, while there is enough evidence to explain the first and the second steps, the supposed oxidative cleavage of bond between $C_{10}$ and $C_{19}$ to afford estrogen and formic acid in the third step is not clear [71]. Blockade of any conversion in the pathway potentially leads to decreased estrogen production, but more specific suppression will result from inhibition of the final step that is unique to estrogen biosynthesis [72]




Scheme (1-5): Mechanism of estrogen production [73].

The aromatase enzyme has a four- to a fivefold higher affinity for androstenedione compared with testosterone [74]. Thus, aromatization of androstenedione into E1 is the major pathway of estrogen synthesis in postmenopausal women. While E1 is inactive by itself with respect to stimulating estrogen receptor activation, it is easily converted to E2 by multiple dehydrogenases [75].

## *1.8 Aromatase inhibition (AIs)*

Aromatase inhibitors have a central role in endocrine therapy for estrogen receptor (ER)-positive breast cancer in postmenopausal women. [76], it interferes with the body's ability to produce estrogen from androgens by suppressing aromatase enzyme activity. Aromatase inhibitors use two distinct mechanisms to block the action of aromatase and thereby reduce estrogen production. Type I inhibitors such as exemestane and formestane (Lentaron) are androgen-like compounds that bind irreversibly to the substrate-complex, causing permanent inactivation of the enzyme [77]. This could potentially lead to prolonged estrogen deprivation even after the drug is cleared. These drugs are also known as aromatase inactivators. Type II inhibitors such as aminoglutethimide, letrozole, and anastrozole are nonsteroidal compounds that reversibly bind to the heme-iron component of the aromatase enzyme, thereby inhibiting the conversion of androgen to estrogen in an indirect fashion [78], figure (1-3) shown chemical structures for both types.

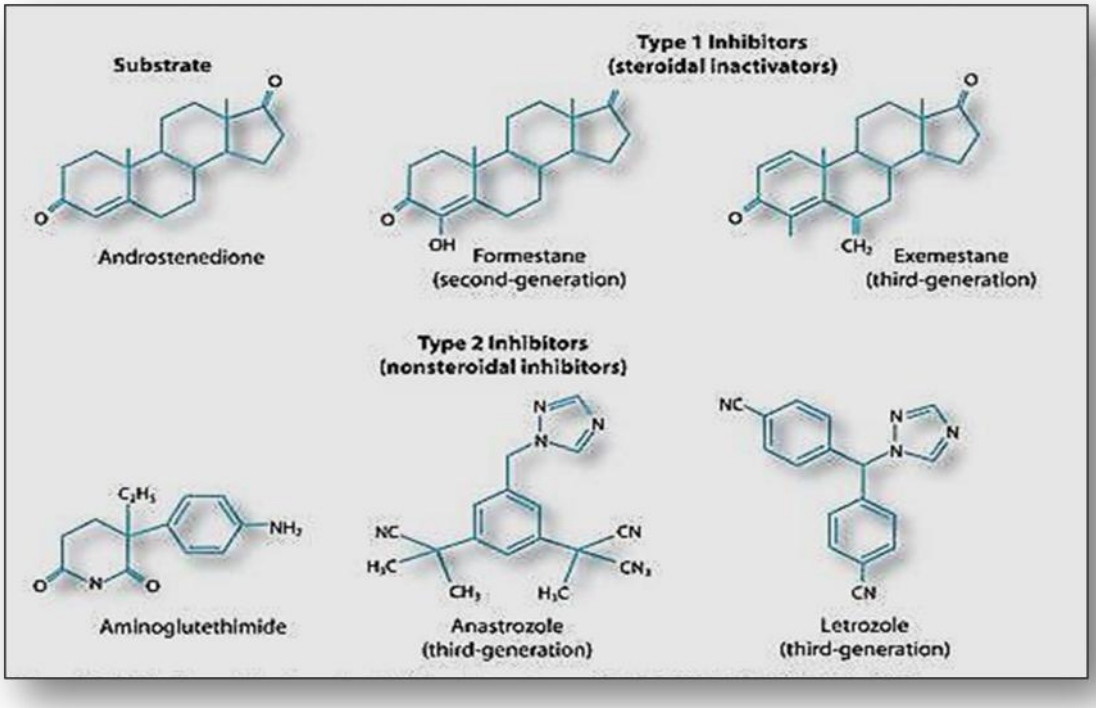
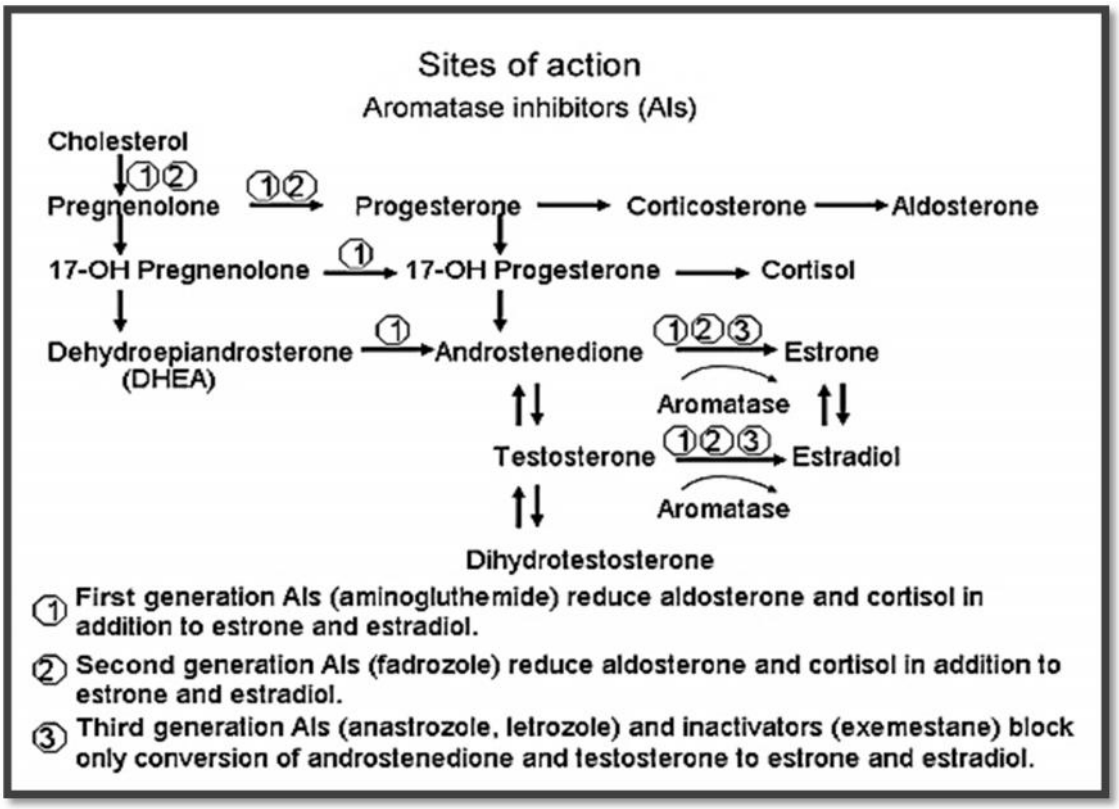


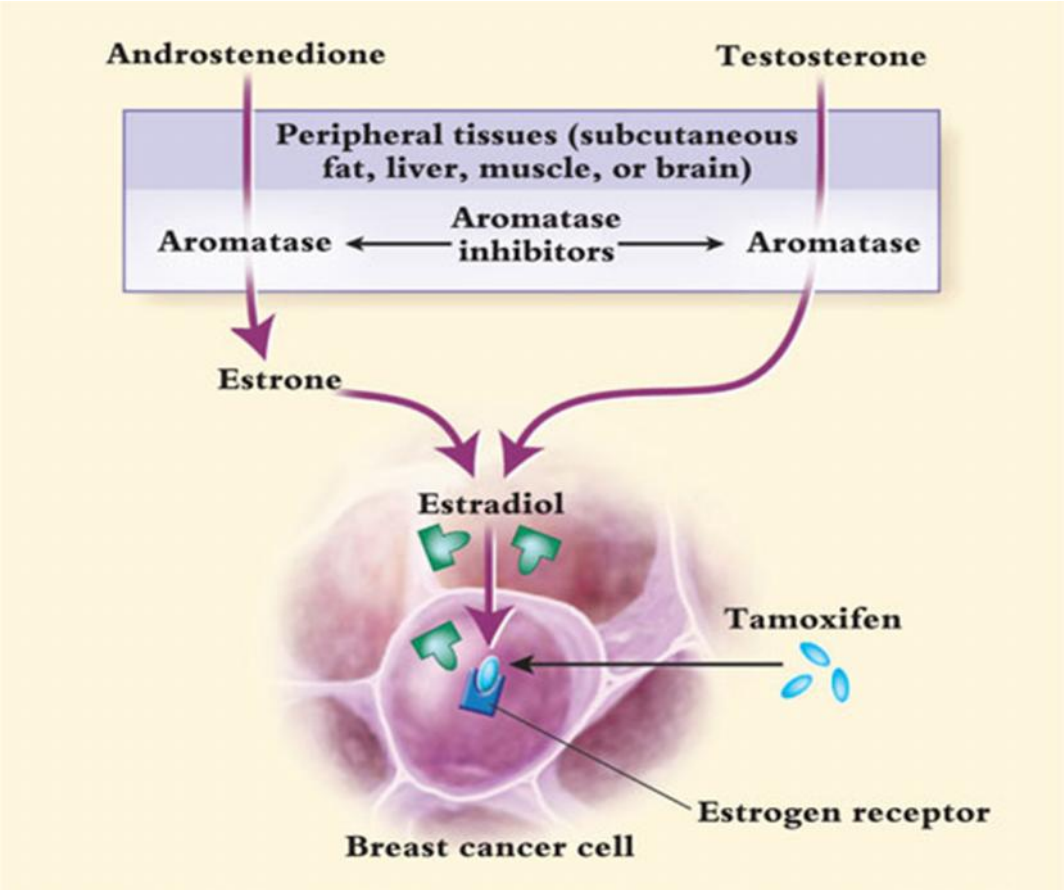

Figure (1-3): Chemical structures of aromatase inhibitors [79].

Three generations of AIs have been developed [80]. Each successive generation has been associated with higher specificity for the aromatase enzyme as shown in scheme (1-6), fewer adverse events, and greater suppression of aromatase activity. The utility of first and second-generation AIs was limited by adverse events, such as rash, fatigue, dizziness, ataxia, nausea and vomiting, as well as by a decrease of enzyme selectivity. Third-generation AIs are superior to earlier versions because they are associated with less adverse events and greater suppression of aromatase activity [81].




Scheme (1-6): Metabolic pathways differentially targeted by aromatase inhibitors (AIs)[81].

The aromatase inhibitors are very specific and selective for the aromatase enzyme and are well tolerated by patients. One of the causes, why aromatase inhibitors are effective second-line therapies, is that their mechanism of action differs from tamoxifen, (scheme (1-7)). Tamoxifen blocks the action of estrogen at the receptor level, whereas aromatase inhibitors block the synthesis of estrogen in peripheral tissues including the breast. However, tamoxifen is a partial estrogen agonist in the breast, which may appear in less than optimal antitumor activity. Anastrozole, letrozole, and exemestane are proving to be effective first-line treatment options after surgery for patients with hormone-dependent advanced breast cancer. In two separate trials, anastrozole was shown to be either equivalent to [82] or superior to tamoxifen [83].




Scheme (1-7): comparison between aromatase inhibitors and tamoxifen[82].

Despite the great interest of aromatase as a drug target against breast cancer detailed structural and spectroscopic information on this enzyme became available only in the past few years. As such, the enigmatic mechanism of the final aromatization step is still a matter of debate [84]

## 1.9 Anastrozole (Arimidex)

chemically known as 2,2'-(5-((1H-1, 2,4-triazol-1-yl)methyl)-1,3-phenylene)bis(2-methylpropanenitrile)[85], is a non-steroidal , third-generation and highly potent aromatase-inhibiting drug approved for treatment of breast cancer after surgery, as well as for metastasis in post-menopausal women [86].

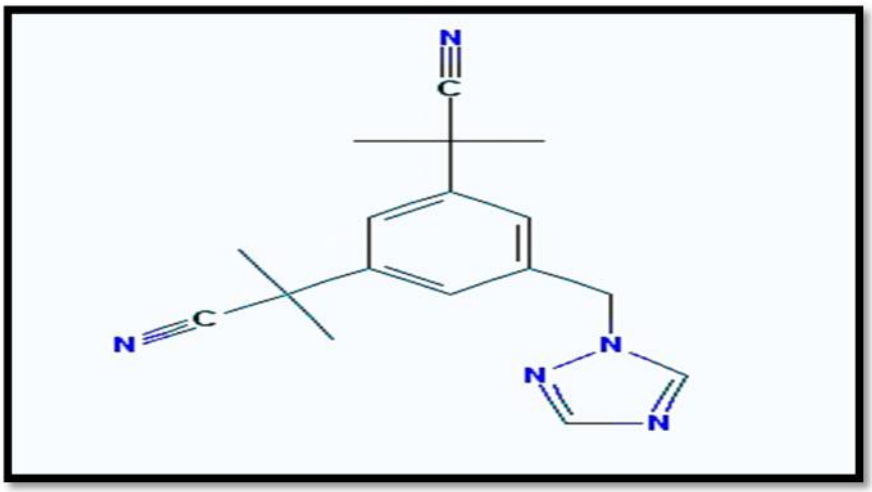
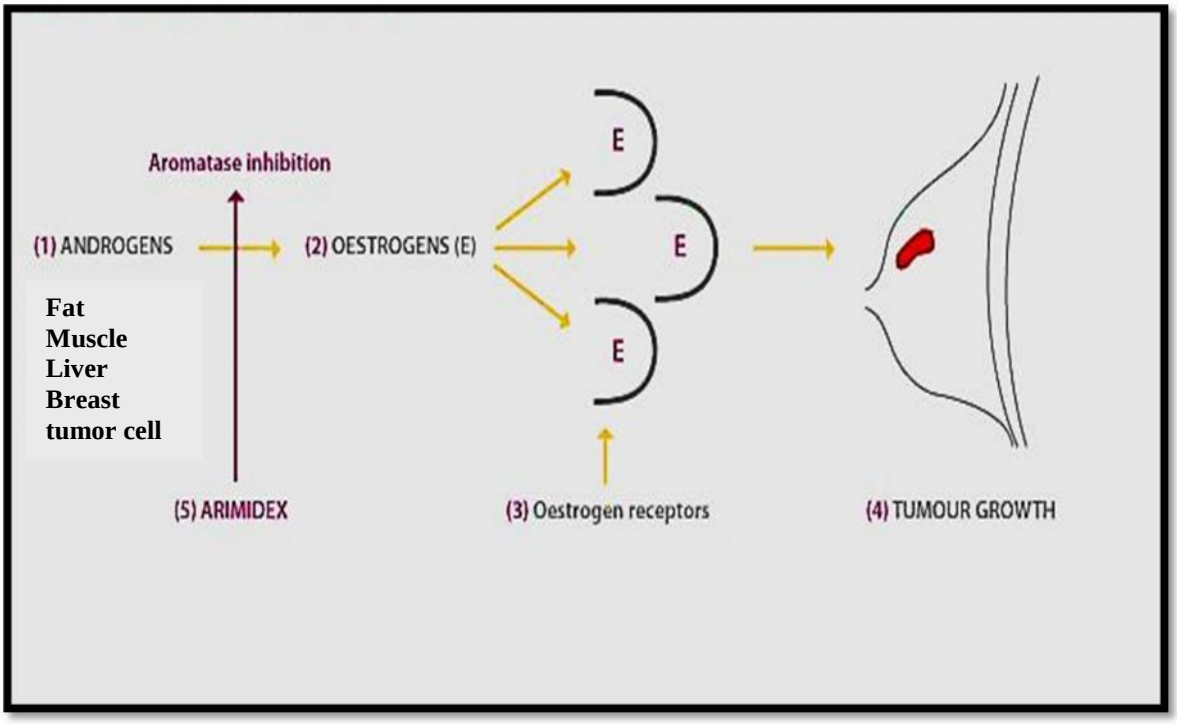



Figure (1-4): anastrozole structure[85].

It is given orally, is well tolerated, and provides almost complete estrogen suppression both systemically and intratumorally, see scheme (1-8) [87]. Anastrozole is effective in treating advanced breast cancer and at a dose of 1 mg once-daily, it significantly increases survival time compared with megestrol acetate [88], Anastrozole has been shown to significantly reduce the rates of breast cancer recurrences. This trial led to the approval of anastrozole by the US Food and Drug Administration for adjuvant treatment of hormone receptor-positive early stage breast cancer [89].




Scheme (1-8): mechanism action of anastrozole[87].

Table (1-2) show compares the degree of aromatase inhibition achieved among drugs of contemporary importance. This demonstrates that while aminoglutethimide and the second-generation inhibitors suppress aromatase by little more than 90% at their clinically used dosages [90], the new third-generation compounds approach complete ablation of aromatase activity. Anastrozole was found to inhibit by greater than 97% in all patients [91].

**Table (1-2):** Degree of Whole-body Aromatase Inhibition by Drugs Used in Breast Cancer Clinical Efficacy [91].

| Drug | Dose | Mean percentage inhibition | Reference |
|---|---|---|---|
| Aminoglutethimide (+ hydrocortisone) | 1000 mg (+ 40 mg)/d | 90.6 | MacNeill *et al.*, 1992 |
| Formestane | 250 mg/2 w (im) | 84.8 | Jones *et al.*, 1992 |
| Fadrozole | 2 mg/d | 82.4 | Lonning *et al.*, 1991 |
| Vorozole | 1 mg/d | 93.0 | Van der Wall *et al.*, 1993 |
| Letrozole | 2.5 mg/d | > 99.1 | Geisler *et al.*, 2001 |
| Anastrozole | 1 mg/d | 97.3 | Geisler *et al.*, 2001 |
| Exemestane | 25 mg/d | 97.9 | Geisler *et al.*, 1998 |

## *1.10 Tumor cell line*

The first human cell line was established in a Baltimore laboratory over 50 years ago by George Gey. This cell line was HeLa – named after Henrietta Lacks, the lady from whom the cell line was derived, who had cervical carcinoma. Gey's vision paved the system for cell culture as we know it today, allowing its widespread evolution into an important experimental tool in cancer research [92]. Cell lines derived from tumours are the most frequently utilized models in cancer research and their use has advanced the understanding of cancer biology tremendously over the past decades [93].

Cell lines are widely used in many aspects of laboratory research and particularly as *in-vitro* models in cancer research. They have a number of advantages; for example, they are easy to handle and represent an unlimited self-replicating source that can be grown in almost infinite

quantities. In addition, they exhibit a relatively high degree of homogeneity and are easily replaced from frozen stocks if lost through contamination. However, there are disadvantages. Cell lines are prone to genotypic and phenotypic drift during their continual culture. This is particularly common in the more frequently used cell lines, especially those that have been deposited in cell banks for many years [94].

A number of factors influencing drug responses in tumor cell lines, such as solubility, chemical or metabolic stability, protein binding, and cellular uptake, can limit drug-induced inhibition of cell growth, knowledge of the relationship between such factors and drug structure facilitates the prediction of activity of new analogues in a series [95]

In 1970, the estrogen receptor-positive MCF-7 cell line (MCF-7 is the acronym of Michigan Cancer Foundation -7, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers) [96], was obtained from a patient with metastatic breast cancer. Since then MCF-7 cell has become a prominent model system for the study of breast cancer as it compares to the susceptibility of the cells to apoptosis [97].

MCF7 is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk [98]. Cancers originating from ducts are acknowledged as ductal carcinomas, while those beginning from lobules are known as lobular carcinomas. Breast cancer occurs in humans and other mammals, while the overwhelming majority of human cases occur in women, male breast cancer can also occur [99]. MCF-7 cells are helpful for *in-vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen, in the form of estradiol, via

estrogen receptors in the cell cytoplasm. This makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line [100].

## 1.11 Cell viability assays (cytotoxicity)

Viability assays are designed to measure activities attributable to cellular maintenance and survival by screening and cytotoxicity tests of chemicals. It uses various reagents for cell viability detection. They are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, Co-enzyme production and nucleotide uptake activity. Many have established methods such as colony formation method, crystal violet method, tritium-labeled thymidine uptake method, MTT and WST methods, which that used for counting the number of live cells [101].

### *1.11.1 MTT* cell proliferation assay

One of the most common colorimetric assay for assessment of the metabolic activity is based on the reduction of yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (tetrazole) to purple formazan by NADH in living cells [102]. However, MTT formazan is insoluble in water, and it forms purple needle-shaped crystals in the cells. Therefore prior to measuring the absorbance, an organic solvent is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving a significant well-to-well error [103].

## *1.12 High content screening (HCS)*

High content screening (HCS) is a method that uses automatic microscopy and image analysis techniques to extract multiple phenotypically relevant measurements at the cellular level [104]. HCS was created in 1996 to offer a new platform that could be used to permit relatively high-throughput screening of cells, in which each cell in an array would be analyzed at a subcellular resolution using multicolored, fluorescence-based reagents for both specificity and sensitivity [105].

High Content Screening (HCS), also known as High Content Analysis (HCA), image cytometry, quantitative cell analysis or automated cell analysis, is an automated method that is used to identify substances that alter the phenotype of a cell in a desired manner. This technology is primarily used in biological research and drug discovery and combines fluorescent microscopy, automated cell calculations and phenotyping using image processing algorithms and informatics tools for the user to make decisions about a treatment [106].

High content screening (HCS) combines the efficiency of high-throughput techniques with the ability of cellular imaging to collect quantitative data from complex biological systems. HCS technology is integrated into all aspects of contemporary drug discovery [107], including primary compound screening, post-primary screening capable of supporting structure–activity relationships, and early evaluation of ADME (absorption, distribution, metabolism, and excretion)/toxicity properties and complex multivariate drug profiling. Recently, high content approaches have been used extensively to interrogate stem cell biology [108].


Figure (1-5): **Array Scan (Thermo Fisher Scientific)[107].**

## *1.13 Aim of study*

Part I :

- Study the influence of aromatase inhibitor (anastrozole) on aromatase enzyme activity in the postmenopausal patient with ER-positive receptors use anastrozole as a treatment in comparison with the enzyme activity in newly diagnosed patient with breast cancer and healthy groups.
- Determination the estradiol concentration to assess the effect of anastrozole on aromatization mechanism.
- Detection the correlation between aromatase activity and estradiol in two groups under study.

Part II :

- Detection the cytotoxic effect of anastrozole on three type of cell (MCF7, HepG3 and PC3).
- Inspect the effect of anastrozole on apoptosis mechanism in MCF7 cell line by HCS techniques.

# CHAPTER TWO

# SUBJECTS, MATERIALS AND METHODS

## 2.1 Equipment and apparatuses

Table (2-1): Instrument and their suppliers

| **Instrument** | **suppliers** |
|---|---|
| Micropipettes (8-12 channel) | Gilson, France |
| Centrifuge | Hittich Universal , Germany |
| Centrifuge | Becton Dickinson, England |
| Timer with alarm | Junghans, Germany |
| ELISA | Micro ELISA system(washer and reader) (Thermo, Germany) |
| Weight scale | Raven equipment limited, England |
| Length scale | Salter ,England |
| Incubator | Gallenkamp, United kingdom |
| freezer | Haas , Saudi Arabia |

Table (2-2): kits and their suppliers

| **Kit** | **suppliers** |
|---|---|
| cytochrome $P_{450}$ | Cuasbio , China |
| Estradiol | Demeditec, Germany |
| MTT | Sigma, USA |
| HCS | Fisher-Thermoscintific, Japan |

## 2.2 Subjects

### 2.2.1 Breast cancer subjects

Sixty postmenopausal female with breast cancer were selected according to positive estrogen receptor test, which were carried out in Oncology Teaching Hospital, Medical City, Al Kadhimiya Teaching

Hospital And Al Yarmouk Teaching Hospital during the period from November 2014 to April 2015.

Patients enrolled in the present study were subdivided into: patients with hormone treatment and patienst without any treatment. The study included (40) Iraqi women were treated with anastrozole (1 mg), group (P2) with the ages range from 45 to 75 year and (20) as a newly diagnosis of breast cancer, group ( P1) with ages ranging from 47 to 75 year.

All clinical examination were carried out by their consultant physicians.

### 2.2.2 Control subjects

For the purpose of comparisons, (20) Iraqi postmenopausal control subjects ( group C) with ages ranging from 47 to 75 year, matched for age, sex and ethnic background ( Iraqi) were included in the study.

Control was selected among subjects that apparently healthy in terms of non-diabetic,non-hypertensive and with no family history breast cancer or other types of cancer. In addition, they had no history of smoking or alcohol consumption.

### 2.2.3 Characteristic of patients and control

Breast cancer patient and control were characterized in terms of age, gender, family history, BMI, WHR, WHtR, Estrogen level and Aromatase activity (cytochrome $P_{450}$) were measured for patients and control.

## 2.3 Material and method

### 2.3.1 Blood Sample

Four milliliters of blood samples were collected from each subject by vein puncture using 5ml disposable syringe between (8 am and 11 A.M). The blood samples were poured in activator

clotting tubes, then were centrifuged at (3000rpm) for 10 min after allowing the blood to clot at room temperature. The sera were frozen at (-20 °c) until the assay day.

## 2.3.2 Anthropometric indices measurments

### 2.3.2.1 Body mass index (BMI)

BMI is a standardized estimation of an individual's relative body fat calculated from his or her weight and height. BMI was expressed as weight in kilograms divided by the square of height measurement in meters (Kg/m2) [109].correlation between BMI and obesity has been reported [110].

$$\textbf{BMI} = \frac{\textbf{Weight Kg}}{\textbf{(Height m)}^2}$$

- BMI from 18.5 up to 24.9 may indicate optimal weight.
- BMI lower than 18.5 the person is underweight.
- BMI from 25 up to 29.9 may indicate the person is overweight.
- BMI from 30 upwards the person is obese.

### 2.3.2.2 Waist-to-hip ratio (WHR)

It is the ratio of the circumference of the waist to that of the hips. It is measure the proportion by which fat is distributed around the torso. WHR is so far the most widely used index of central fat distribution and widely used due to its benefits in routine monitoring and assessment in patients [111].

$$\textbf{WHR} = \frac{\textbf{Waist (cm)}}{\textbf{Hip (cm)}}$$

(Ratio of ≥0.85 indicates central obesity).

### 2.3.2.3Waist-to-height ratio (WHtR)

WHtR is another anthropometric index of abdominal adiposity marker; it is a better predictor of metabolic and cardiovascular risk than BMI and WHtR [112].

$$\textbf{WHtR} = \frac{\textbf{Waist (cm)}}{\textbf{Height (cm)}}$$

(Ratio of ≥0.55 highlights cardiovascular disease risk).

## 2.4 Human cytochrome $P_{450}$ 19A1 (CYP19A1) ELISA kit

### 2.4.1 Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for CYP19A1 has been pre-coated onto a micro plate. Standards and samples are pipetted into the wells with a Horseradish Peroxidase (HRP) conjugated antibody specific for CYP19A1. Following a wash to remove any unbound reagent, a substrate solution is added to the wells and color develops in proportion to the amount of CYP19A1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

### 2.4.2 Reagent composition

Table (2-3) : Reagents and their Quantity

| Reagents | Quantity |
|---|---|
| **Standard** | 6 (lyophilize) |
| **HRP-conjugate** | 1 x 6 ml |
| **Wash Buffer (20 x concentrate)** | 1 x 15 ml |

| | |
|---|---|
| **Substrate A** | 1 x 7 ml |
| **Substrate B** | 1 x 7 ml |
| **Stop Solution** | 1 x 7 ml |

### 2.4.3 Assay Procedure

All reagents and samples were Bring to room temperature before use. The sample was Centrifuge again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

1. Fifty µls of standard or sample were added per well.
2. Fifty µls of HRP-conjugate was added to each well. The well was mixed and then incubated for 1 hour at 37°C.
3. Microliter wells were rinsed 3 times with wash buffer (200µl), the plate was stand for 10 seconds, then the remaining wash buffer was removed by aspirating or decanting. Inertly the plate and blotting it against clean paper towel.
4. Fifty µls of substrate a and substrate b were added to each well, mixed well and incubated for 15 minutes at 37°c in the dark.
5. Fifty µls of stop solution was added to each well, gently tap the plate to ensure thorough mixing.
6. The absorbance of each well was a measurement at 450 nm with a microtiter plate reader, within 10 minutes after adding the stop solution.

### 2.4.4 Calculation

The concentration of cytochrome $P_{450}$ (pg/ml) was calculated from the standard curve equation as show (fig. 2.1).

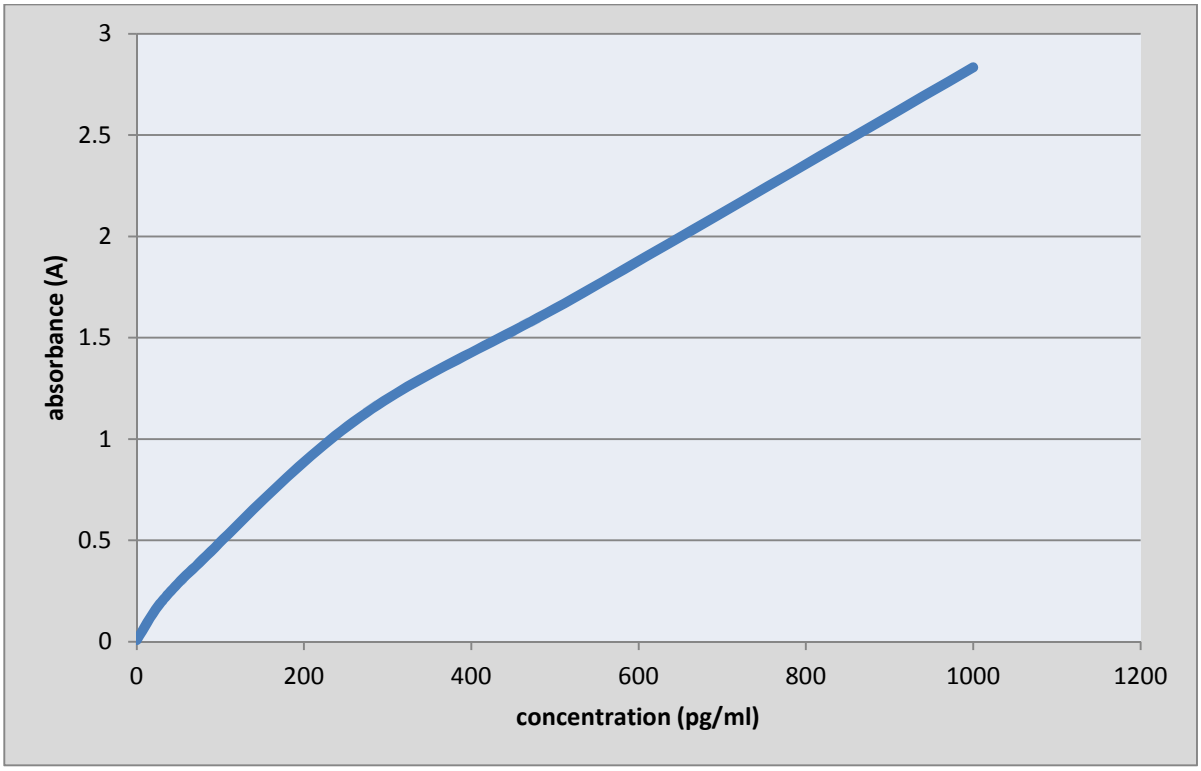



Figure (2-1): cytochrome $P_{450}$ standard curve.

### 2.5.1 Estradiol ELISA kit

### 2.5.1 Principle of the assay

The Estradiol ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtiter wells are coated with a polyclonal [rabbit] antibody directed towards an antigenic site on the Estradiol molecule. Endogenous Estradiol of a patient sample competes with an Estradiol-horseradish peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is inversely proportional to the concentration of Estradiol in the sample. After addition of the substrate solution, the intensity of colour developed is inversely proportional to the concentration of Estradiol in the patient sample.

## 2.5.2 Reagent composition

Table (2-4): Reagent, materials and their Quantities

| Reagents | materials | Quantity |
|---|---|---|
| Standard | 0.03% Proclin 300 + 0.005% gentamicin sulfate | 7 vials |
| estradiol-conjugate | 0.03% Proclin 300, 0.015% 5-bromo-5-nitro-1,3-dioxane (BND) and 0.010% 2-methyl-2H-isothiazol-3-one (MIT) | 25 x 6 ml |
| Wash Buffer (40 x concentrate) | deionized water | 1 x 30 ml |
| Substrate | Tetramethylbenzidine (TMB). | 1 x 14 ml |
| Stop Solution | 1 N acidic solution. | 1 x 14 ml |

### 2.5.3 Assay Procedure

1. A volume of 25 µls from each standard, control and samples with new disposable tips were added into appropriate wells.
2. Two hundred µls of enzyme conjugate was added to each well with thorough mix for 10 seconds (It is important to have a complete mixing in this step) and incubated for 120 minutes at room temperature (without covering the plate). The contents of the wells were briskly shaken out.
3. The wells were rinsed 3 times with diluted wash solution (400 µl per well) and the wells were striked sharply on absorbent paper to remove residual droplets.
4. One hundred µls of Substrate Solution were added to each well and Incubated for 15 minutes at 25°c.
5. Finally, fifty µls of stop solution were added to each well to stop the enzymatic reaction.

6. The absorbance of each well was a measurement at 450nm with a microtiter plate reader, within 10 minutes after adding the stop solution.

### 2.5.4 Calculations

- The absorbance values for each set of standards, controls and patients samples were calculated.
- A standard curve was constructed by plotting the absorbance obtained from each standard against their concentration with absorbance value on the vertical(Y) axis and concentration on the horizontal (X) axis.
- from the standard curve, the corresponding concentration of estradiol is determined by using the absorbance value for each sample

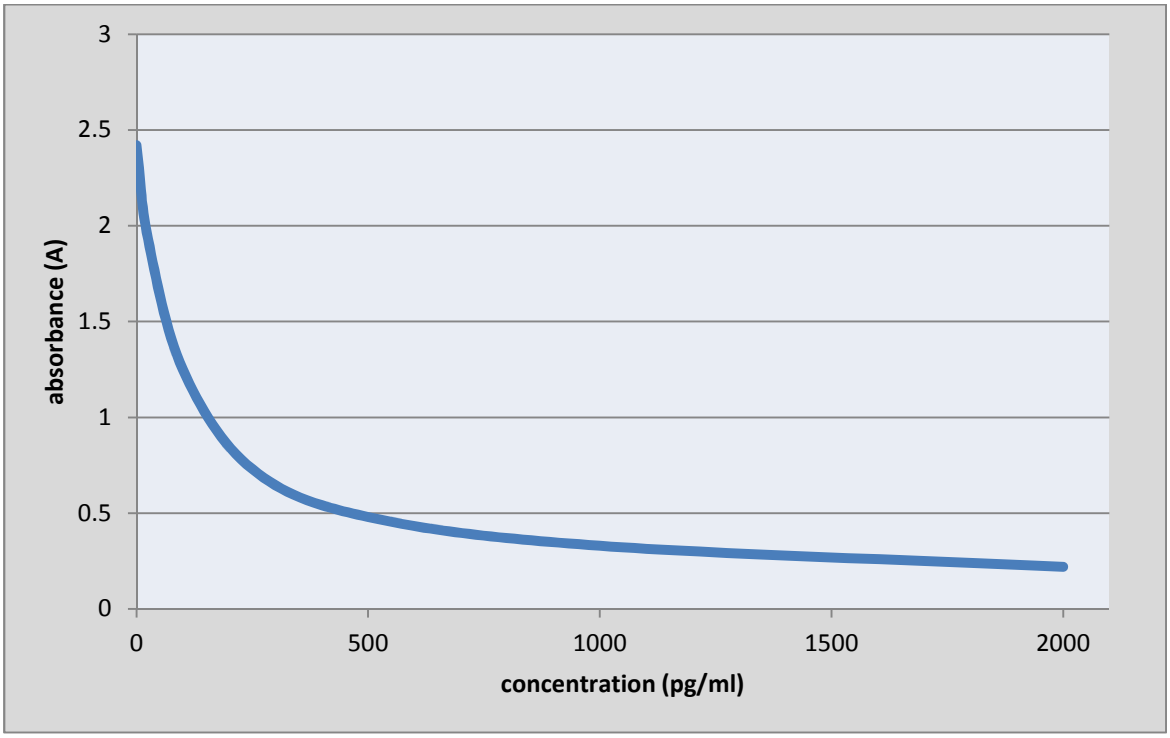



Figure (2-2): estradiol standard curve.

# 2.6 Biological activity

MTT assay and HCS were carried out in the Centre of Biotechnology Research's, Al-Nahrain University, Baghded and Centre for Natural Product Research and Drug Discovery, Department of Pharmacology, Faculty of Medicine, University of Malaya- Malaysia., Respectively.

## 2.6.1 Cytotoxicity Assay (MTT assay)

### 2.6.1.1 Kit contents

- Hidex Chamelon plate Reader with a 570nm filter.
- MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MW = 414), 10 vials, each containing 5 mg
- Multi-channel pipette (8 to 12 channel )
- Pipette tips (10-100µL)
- phosphate-buffered saline (PBS), sterile HCl, 0.01 M solution dimethylsulfoxide (DMSO) optional.
- $CO_2$ incubator (5%).

### 2.6.1.2 Preparation Instructions

- MTT Solution: 5 mg/ml MTT in PBS. Solution must be filter sterilized after adding MTT.
- MTT Solvent: 4 mM HCl, 0.1% all in isopropanol.

### 2.6.1.3 Procedure

1. The tested compound was dissolved in DMSO to prepare the stock solution and serial dilutions were prepared (25 *µ*g/ml–400 *µ*g/ml) by adding 100 µl of the stock solution to culture cellular models

(MCF7 cell line) breast cancer, (hep G2) liver hepatocellular cancer cell (PC3) prostate cancer cell.

2. In each plate, doxorubicin was included as control.
3. After 24 hour in $CO_2$ incubator, MTT (5 µg/mL) was added to each well and the plates were incubated in $CO_2$ incubator for 1-4 hours.
4. Media was removed and DMSO was added into each well to solubilize the formazan crystals.
5. The absorbance was read at wavelength of 570 nm using a micro plate reader.

## 2.6.2 High-Content Screening

## 2.6.2.1 Cytotoxicity 3 Kit

### 2.6.2.2 Kit Contents

- Cytochrome c Primary Antibody
- DyLight™ 649 Conjugated Goat Anti-Mouse IgG
- Mitochondrial Membrane Potential Dye
- Permeability Dye
- Hoechst Dye
- Wash Buffer (10X Dulbecco's PBS)
- Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton® X-100)
- Blocking Buffer (10X)
- Thin Plate Seal Assembly

### 2.6.2.3. Solution Preparation (per 96-well plate)

| | |
|---|---|
| 1X Wash Buffer | 20 ml of 10X Wash Buffer was added to 180 ml ultrapure water. Store buffer at 4°C for up to 7 days. |
| Fixation Solution | 3 ml of 16% paraformaldehyde solution was added to 9 ml of 1X Wash Buffer just before its used . |
| 1X Permeabilization Buffer | 1.5 ml of 10X Per meabilization Buffer was added to 13.5 ml of the 1X Wash Buffer. Stored at 4°C for up to 7 days. |
| 1X Blocking Buffer | 5 ml of 10X Blocking Buffer was added to 44 ml of 1X Wash Buffer. Stored at 4°C for up to 7 days. |
| Primary Antibody Solution | 15 µl of the Cytochrome c Primary Antibody was added to 6 ml of 1X Blocking Buffer. solution prepared just before each assay. |
| Secondary Antibody/ Staining Solution | 0.6 µl of Hoechst Dye and 12 µl of the DyLight 649 Goat Anti-Mouse was added to 6 ml of 1X Blocking Buffer. solution prepared just before each assay. |
| Live Cell Staining Solution | 117 µl of DMSO was added to the Mitochondrial Membrane Potential Dye to make a 1 mM stock solution. Just before use, 2.1 µl of Permeability Dye and 21 µl of Mitochondrial Membrane Potential Dye was added to 6 ml complete medium pre-warmed to 37°C. |

### 2.6.2.4 Procedure

1. Dilute solutions of anastrozole at different concentration (200, 100, 50, 25 µg/ml) was prepared and 25 µl was added to the cells. cells Incubated at 37°C for 24 hours.
2. Fifty µl of live cell staining solution was added to each well.
3. The cells were incubated at 37°C for 30 minutes.
4. Gently the medium and the staining solution and 100 µl/well of fixation solution and was plate incubated for 20 minutes at room temperature.
5. Gently the fixation solution was aspirated and 100 µl/well of 1x wash buffer was added.

6. Wash Buffer was removed and 100 µl/well of 1X permeabilization buffer was added and incubated for 10 minutes at room temperature protected from light.
7. Permeabilization buffer was aspirated and washed plate twice with 100 µl/well of 1X wash buffer.
8. Wash buffer was Aspirate and 100 µl of 1X blocking buffer was added and incubated for 15 minutes at room temperature.
9. Blocking Buffer was aspirated and 50 µl/well of primary antibody Solution was added . Incubated for 60 minutes protected from light at room temperature.
10. Primary antibody solution was aspirated and washed plate three times with 100 µl/well 1x wash buffer.
11. Wash Buffer was aspirated and 50 µl/well of Secondary Antibody/Staining Solution was added. Incubated for 60 minutes protected from light at room temperature.
12. secondary antibody/staining solution was aspirated and washed plate three times with 100 µl/well of 1x wash buffer.
13. One hundred µl/well of 1x wash buffer was added.
14. Plate was Sealed and evaluated on the array scan hcs reader.
15. Sealed plates was Stored in dark at 4°C. The plates were evaluated within 24 hours after assay completion.

## 2.7 statistical analyses

Statistical package for The Social Sciences (SPSS), version 17.1 for windows software (SPSS Inst. Inc., Chicago, USA) was used for statistical analysis. The data normally distributed and were expressed as mean ± standard deviation (SD). Student's *t*-test were performed to analyze the statistical significance of difference between group (C ) and group (P).

One-way analysis of variance (ANOVA) hoc test was used to compare the parameters among groups (C ), (P1) and (P2) followed by post hoc test.

A difference among groups was defined to be statistically significant if the corresponding p-value was less than 0.05. Correlations between variable were determined by person correlation coefficients (r-value) .

Least significant difference ( LSD) to compare between means in this study.

# CHAPTER THREE

# RESULTS AND DISCUSSION

## Results and discussion

The results presented in this chapter were based on the analysis of 60 patients with an established diagnosis of breast cancer in comparison with control group of 20 apparently healthy control subjects.

## 3.1 Characteristics of the patients and selecting parameters

Sixty Iraqi women with breast cancer blood samples were included in this study. The (mean±SD) age of the patients were (58.15±7.45) years old; ranging from (45-75) years old; seven cases were under (50) years old (11.66%); twenty-six cases between (50-59) years old about (43.33%); twenty-three cases were between (60-69) years old about (38.33%) and four cases above (70) years old about (6.66%).

While the (mean ±SD) age of control (58.55±6.85) ranging from (48-75) years old ; two cases were under (50) years old (10%); eleven cases between (50-59) years old about (55%); five cases were between (60-69) years old about (25%) and two cases above (70) years old about (10%).as shown in the figure and table (3-1).

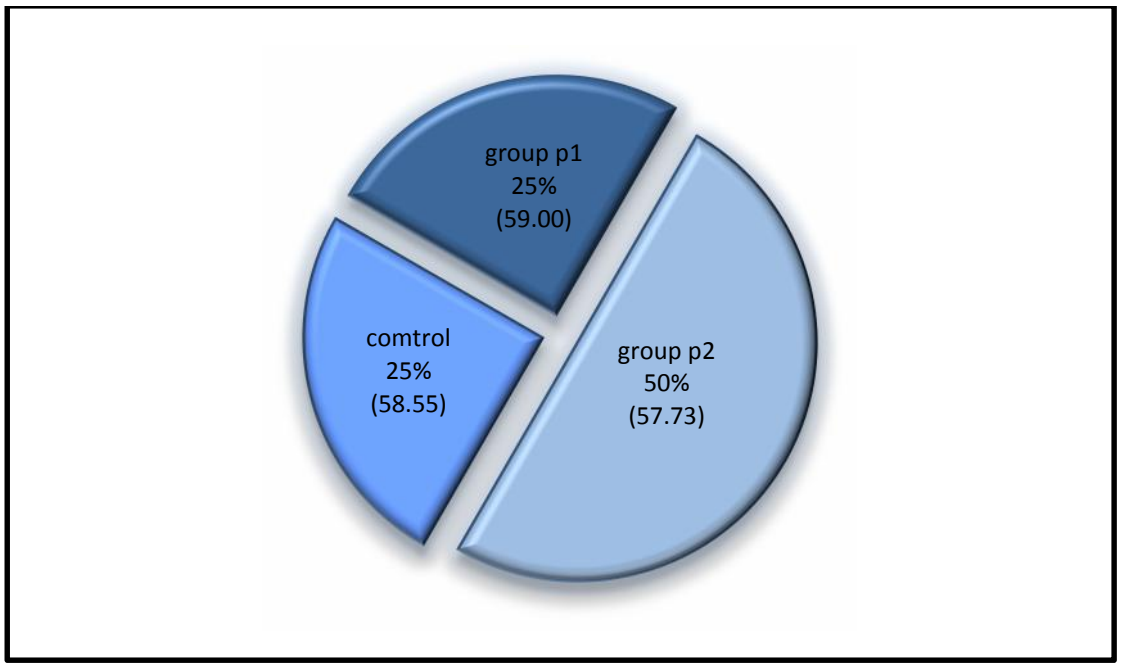



Figure (3-1): Distribution of studying groups.

### 3.1.1 Family history:

Women with a family history of breast cancer, especially in a first-degree relative (mother, sister or daughter), are at high risk of developing breast cancer; Compared to women without a family history, risk of breast cancer are more than 1.8 times higher for women with one first-degree female relative that has been diagnosed [113], and that was agreed with this study which included 60 cases, 41 patients (68.33%) with breast cancer and has a family history and 19 (31.66%) of them had no family history.

## 3.1.2 Anthropometric indices

The result of mean weight (79.55 vs. 66.90), height (158.61 vs. 161.90), BMI (31.51 vs. 25.44) and waist (105.61 vs. 102.8), showed a significant increase of breast cancer patients group (P) ($P$=0.001*)*, *(*$P$=0.001*), (*$P$=0.001*)* and *(*$P$=0.036*)* respectively when compared with healthy control group (C), shown in table (3-1) and figure (3-2). In contrast, the mean of WHR and WHtR were (0.95 and 0.66) respectively in the group (P) showed no significant differences ($P \geq 0.05$), when compared with the group (C) (0.96 and 0.63)(Table 3-1).

Overweight and obesity are defined as abnormal or excessive fat accumulation as measured by the body mass index (BMI), the results of the present study provide strong support for a positive association (highly statistically significant (P=0.00)) between body fat and breast cancer risk in postmenopausal women shown in the table (3-1). The extensive study agrees with the result by confirmed a direct association between BMI with the risk of developing the disease, Karim S.[114]. Other study found that the BMI-breast cancer association is stronger for estrogen receptor positive/progesterone receptor-positive (ER+PR+) tumors (33% increases per 5 $kg/m^2$ increment for postmenopausal breast cancer), De Pergola[115].

The clinical evidence suggests the association of breast cancer with general obesity is stronger than the association with central obesity in a postmenopausal woman by indicating a significant difference when compared with control group; this result is in agreement with Rohan T. [116].

**Table** (3-1): mean (±SD) of Age, BMI, WHR, WHtR and family history in group (C) and group (P)

| Parameter | Group (C) (n=20) | Group (P) (n=60) | *P* – value |
|---|---|---|---|
| **Age (year)** | 58.55±6.85 | 58.15±7.45 | N.S |
| **Weight (Kg)** | 66.90±8.29 | 79.55±15.85 | 0.001 |
| **Height (cm)** | 161.90±4.81 | 158.61±3.44 | 0.001 |
| **BMI ($Kg/m^2$)** | 25.44±2.24 | 31.51±5.71 | 0.00 |
| **Waist (cm)** | 102.8±8.78 | 105.61±11.95 | 0.036 |
| **Hip(cm)** | 106.65±7.04 | 110.20±10.31 | N.S |
| **WHR** | 0.96±0.06 | 0.95±0.08 | N.S |
| **WHtR** | 0.63±0.4 | 0.66±0.06 | N.S |
| **Family history No.(%)** | _____ | 41 (68.33%) | ____ |

$P \leq 0.05$: significant, N.S. ($P > 0.05$): No significant.

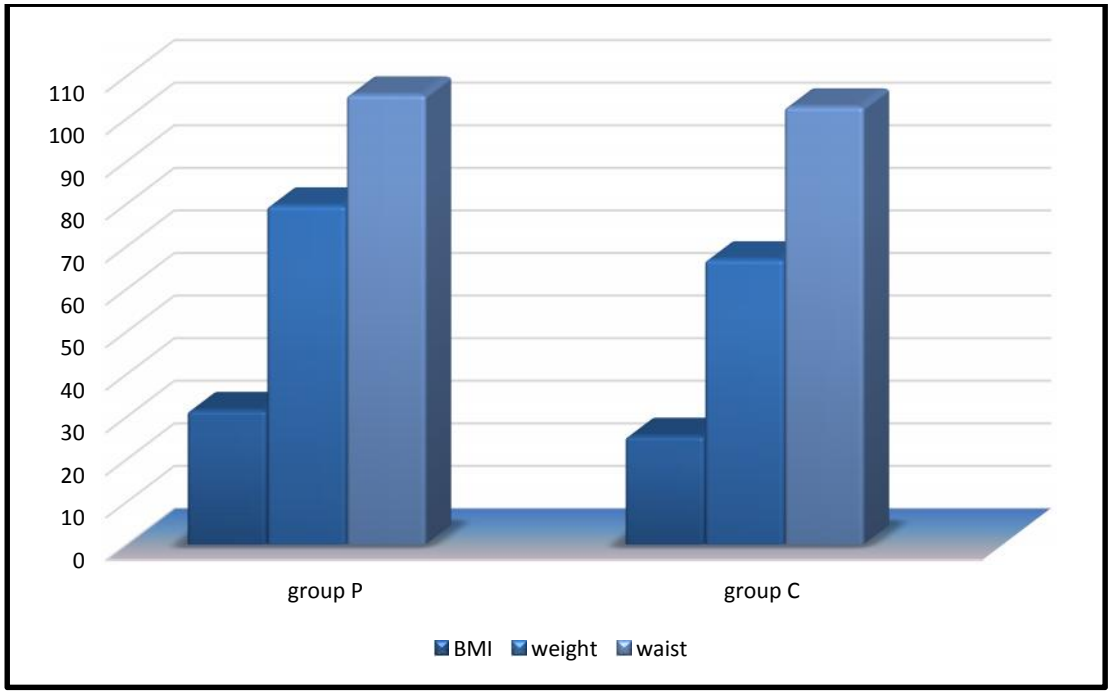



Figure (3-2) mean distribution of weight, BMI and waist in studied group patient and control.

### 3.1.3 Level of serum estradiol (E2)

The results of serum estradiol level for breast cancer patients group and control group were shown in table ( 3-2 ), figure (3-3) in which mean estradiol was found to be elevated and significantly in breast cancer patients group (P) as compared with non-breast cancer control group (C),(14.76 vs. 12.29)(*P*=0.004*).*

Current results agrees with Atoum M.[117] who revealed that an increase in estradiol during the years when women haven't the functional ovaries really cause breast cancer, a hypothesis which proposed that estrogen might play an important role in affecting breast cancer risk.

This association between estrogen and breast cancer can be explained by two hypotheses: 1) the binding between estrogen and ER enhances cell proliferation of mammary cells, and increase in cell division and DNA synthesis elevates the risk for replication errors that may result in

mutations. 2) Estrogen metabolism leads to the production of genotoxic byproducts that could damage DNA, leading to point mutations, Rehmani, N. *et al*., 2015[118].

**Table (3-2):** Mean (±SD) level of serum estradiol of group (C) and group (P).

| Parameter | Group (C) (n=20) | Group (P) (n=60) | *P* _ value |
|---|---|---|---|
| Estradiol pg/ml | 12.29±1.86 | 14.76±3.4 | 0.004 |

*P* ≤ 0.05: significant, N.S. (*P* > 0.05): No significant.

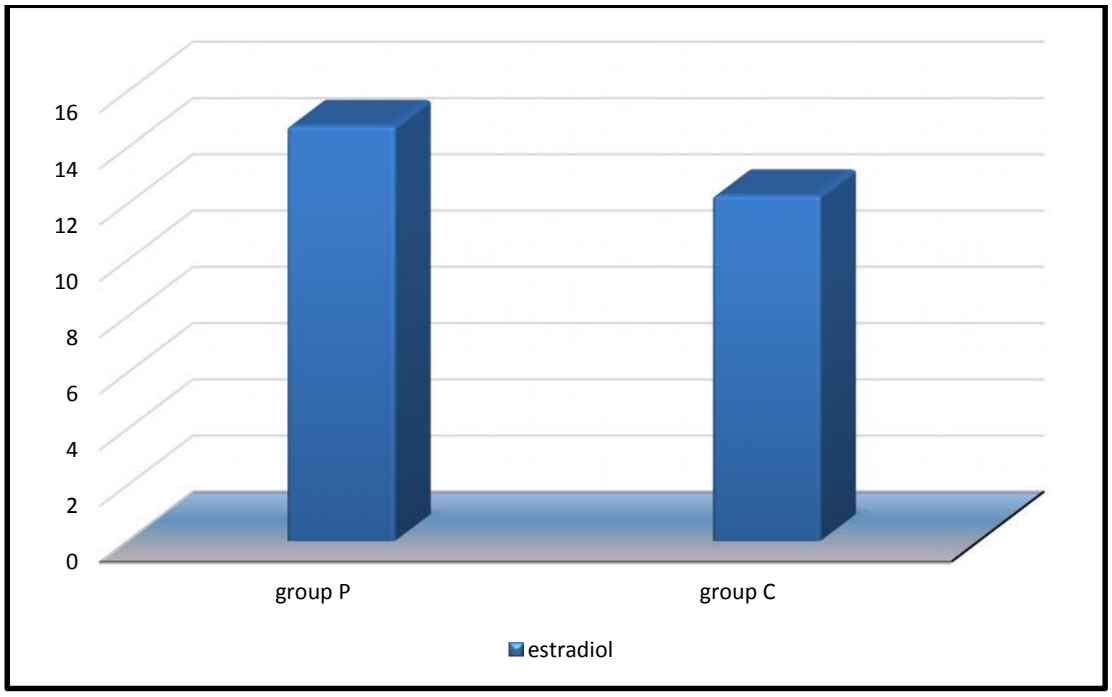



Figure (3-3) mean distribution of estradiol in studied group patients and control.

### 3.1.4 Level of serum Aromatase

The result of serum aromatase for breast cancer patients group and control group as shown in table (3-3)and figure (3-4) showed that mean level of aromatase was found to be significantly higher in breast cancer patients group (P) when compared with non-breast cancer control group (C), (487.133 vs. 347.97) (P=0.006)

The reason for the elevated and significant levels of aromatase in patients group as compared with control group is due to the increasing of aromatization mechanism of androgen to provide more necessary estrogen for tumor growth that was compatible with Bhatnagar, S. [119]. Who elucidate that aromatase is the enzyme responsible for the synthesis of estrogens, and estrogens play a major role in the development of breast cancer. Abnormal expression of aromatase in breast cancer cells and/or surrounding adipose stromal cells, especially in postmenopausal women, may have a significant influence on breast tumor maintenance and growth in breast cancer patients.

**Table (3-3):** Mean (±SD) level of serum aromatase of group (C ) and group(P).

| Parameter | Group (C) (n=20) | Group (P) (n=60) | P – value |
|---|---|---|---|
| Aromatase pg/ml | 347.97±73.17 | 487.133±214.39 | 0.006 |

$P \leq 0.05$: significant, N.S. ($P > 0.05$): No significant

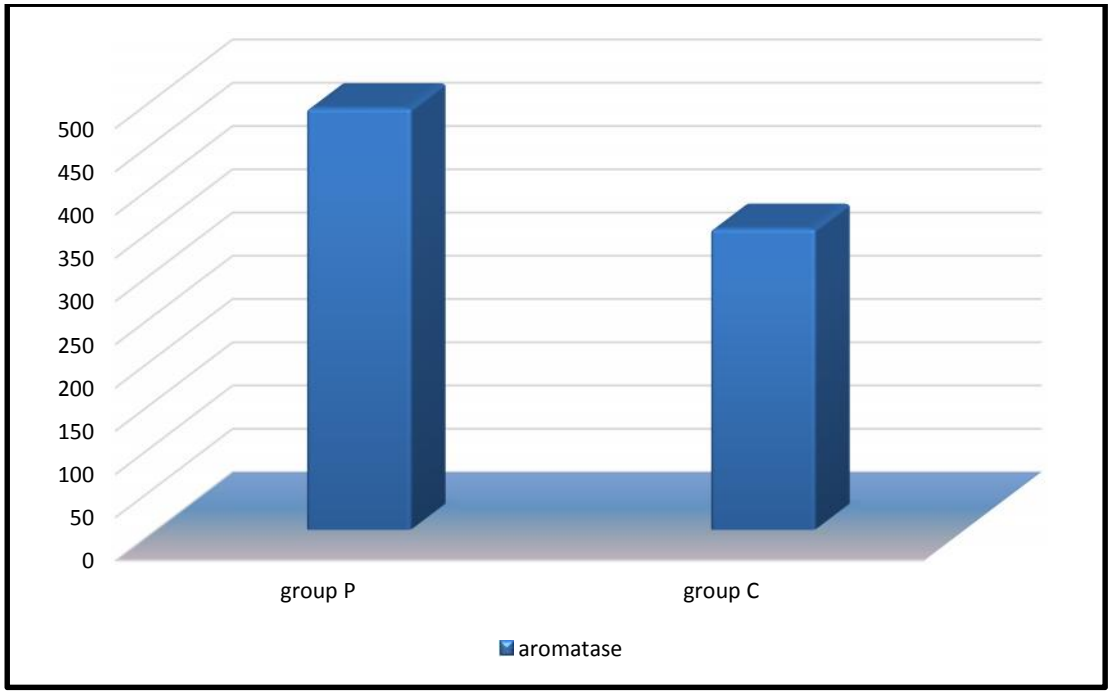



Figure (3-4) mean distribution of aromatase in studied group patients and control

## 3.2 ANOVA analysis

To investigate the effect of anastrozole on estradiol and aromatase activity in patients with breast cancer and study if this marker can be considered to be a predictor of progression of breast cancer with hormone sensitivity. Group (P) was sub- grouped according to with treatment (P2) and without treatment (P1)

### 3.2.1 Anthropometric indices

The result of mean weight (78.25and 82.15 vs. 66.90), height (158.55 and 158.75 vs. 161.90) and BMI (30.98 and 32.56 vs. 25.44), showed significantly increase in two breast cancer patients group (P2) and (P1) ($P$=0.003*), (*$P$=0.006) *and (*$P$=0.000*)* respectively when compared with healthy control group (C), shown in table (3-4) and figure (3-5).

Furthermore, BMI showed a significant difference when compared each group with control (P<0.001). Postmenopausal women with increased BMI are at greater risk of developing breast cancer due to increased estrogen production in the adipose tissue. These explain why women with higher BMI were more likely to have hormone receptor-positive breast tumors, Yamashita, T. [120].

The results are in disagreement with Artac, M.[121] who found that a significant differences in WHR and WHtR with increased risk of postmenopausal breast cancer.

**Table (3-4)**: mean (±SD) level of Age, BMI, WHR, WHtR, duration of treatment and family history in group (C), group (P1) and group (P2)

| Parameter | Group (C) (n=20) | Group (P1)(n=20) | Group (P2)(n=40) | *P*-value |
|---|---|---|---|---|
| Age (year) | 58.55±6.85 | 59.00±7.87 | 57.73±7.29 | N.S |
| Weight (Kg) | 66.90±8.29 | 82.15±16.07 a** | 78.25±15.78 b* | 0.003 |
| Duration of treatment (month) | ____ | ____ | 12.82±9.39 | |
| Height (cm) | 161.90±4.81 | 158.75±3.04 a* | 158.55±3.67 b** | 0.006 |
| BMI ($Kg/m^2$) | 25.44±2.24 | 32.56±6.24 a*** | 30.98±5.43 b*** | 0.000 |
| Waist (cm) | 102.8±8.78 | 106.30±12.79 | 105.27±11.65 | N.S |

| | | | | |
|---|---|---|---|---|
| Hip(cm) | 106.65±7.04 | 110.75±9.78 | 109.92±10.67 | N.S |
| WHR | 0.96±0.06 | 0.96±0.11 | 0.95±0.06 | N.S |
| WHtR | 0.63±0.04 | 0.66±0.07 | 0.66±0.06 | N.S |
| Family historyNo. (%) | _____ | 11(55%) | 30(75%) | |

*P** ≤0.05; *P***<0.01; *P****<0.001; no asterisk: *P* > 0.05.

a) Indicate significant difference between groups (C) and (P1).

b) Indicate significant difference between groups (C) and (P2).

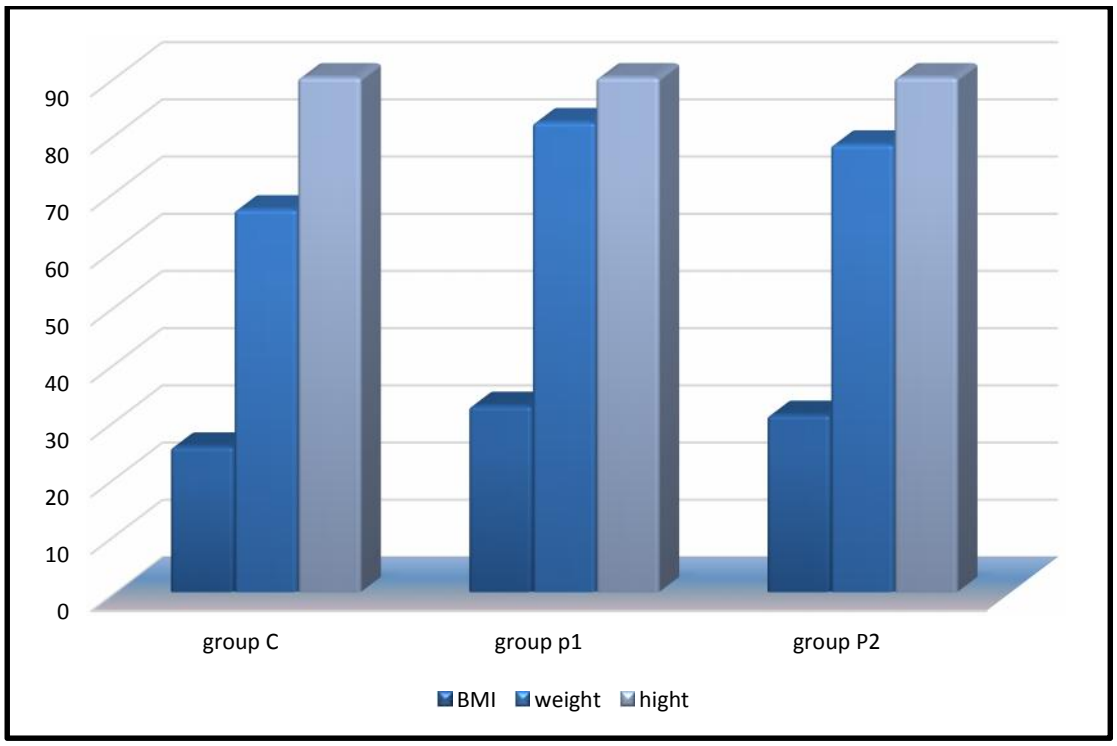



Figure (3-5) mean distribution of weight, BMI and height in studied groups (P1),(P2) and control

## 3.2.2 Level of serum estradiol

The result of estradiol level in serum samples for two different breast cancer groups and control group as shown in the table (3-5) and figure (3-6), showed significant differences between groups (C), (P1) and (P2)(P=0.00). A significant increase in the mean of estradiol was observed in the group (P1) in comparison with that of the group (C) (18.10 vs. 12.29)(P<0.001). A significant increase in the mean of estradiol was also observed in the group (P1) comparison with a group (P2) (18.10 vs. 13.09) (P< 0.001).

Some breast cancer cells require estrogen for growth and if deprived of this hormone, will regress. Consequently, estrogen deprivation therapy is a major treatment strategy for hormone-dependent breast cancer. There are various forms of endocrine therapy, but recently inhibiting the aromatase enzyme, which catalyzes the conversion of androgens to estrogen, have been increasingly used, Miller R.[122]. Among these aromatase inhibitors available, anastrozole has a well emphatic role in clinical practice for advanced disease and first-line treatment. Given that aromatase inhibitors act by the suppression of estrogen synthesis and withdrawal of estrogenic growth support to ER-positive breast cancer Behan, L. A.[123]. It was reasonable to hypothesize that the efficacy of anastrozole on estradiol has appeared in a group (P2) (13.09 pg/ml) in the table (3-5). The results is in agreement with the previous literature, To Q.[124] regarding the role of aromatization in postmenopausal breast cancer patients, which suggest that aromatase inhibitors can greatly reduce estrogen concentrations, hence avoiding stimulation of ER-positive breast cancer cells Dowsett, M. et al. 2010[125] and Kyvernitakis I. [126].

Table (3-5**):** Mean (±SD) level of serum estradiol of group (C), group (P1) and group (P2).

| Parameter | Group(C)(n=20) | Group(P1)(n=20) | Group(P2)(n=40) | *P* – value |
|---|---|---|---|---|
| Estradiol pg/ml | 12.29±1.86 | 18.10±3.9 a*** | 13.09±1.44 c*** | 0.000 |

*P** ≤ 0.05; *P***<0.01; *P****<0.001; no asterisk: *P* > 0.05.

a) Indicate significant difference between groups (C) and (P1)

c) Indicate significant difference between groups (P1) and (P2)

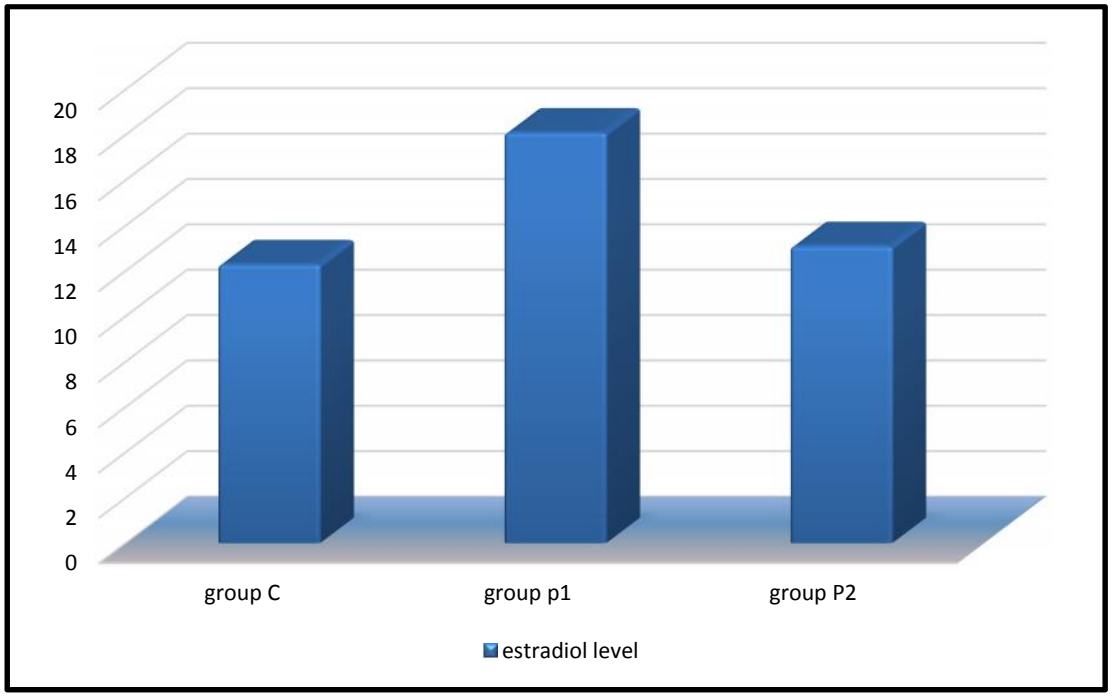



Figure (3-6) : comparison of the mean estradiol between the three groups (C),(P1) and (P2).

### 3.2.3 Level of serum Aromatase

The result of aromatase level in serum sample for two different of breast cancer groups and control group as shown in table and figure (3-6) (3-7)respectively, showed significant differences between groups (C), (P1) and (P2)(P=0.00). A significant increase in the mean of aromatase was observed in the group (P1) in comparison with that of the group (C) (746.24 vs. 347.97) (P< 0.001). A significant increase in the mean of aromatase was also, observed in group (P1) comparison with a group (P2) (746.24 vs. 452.34) (P< 0.001).

Increasing evidence suggests that non-steroid anastrozole are highly specific, potent and have less adverse effects during breast cancer treatment, Milani M. [127], given the mechanism of action of anastrozole that inhibits the aromatase enzyme by non-covalent binding to the aromatase enzyme heme moiety and preventing androgen binding by saturating the binding site, are a successful as a first-line therapy in postmenopausal women, Ji J.[128].

The results of these studies demonstrate that anastrozole is potent in reducing the aromatase activity which appears clearly in the group (P2) when compared with a group (P1). Also, agreement with Geisler j. and Smith, I. [139,130], which revealed the anastrozole was able to block aromatase activity with high efficiency as shown in the group (P2).

**Table (3-6):** Mean (±SD) level of serum aromatase of group (C ) ,group (P1) and group (P2).

| **Parameter** | Group(c)(n=20) | Group(p1) (n=20) | Group(p2) (n=40) | *P*–value |
|---|---|---|---|---|
| Aromatase pg/ml | 347.97±73.17 | 746.24±170.86 a*** | 452.34±198.22 c*** | 0.000 |

*P** ≤0.05; *P***<0.01; *P****<0.001; no asterisk: *P* > 0.05.

a) Indicate significant difference between groups (C) and (P1)

c) Indicate significant difference between groups (P1) and (P2)

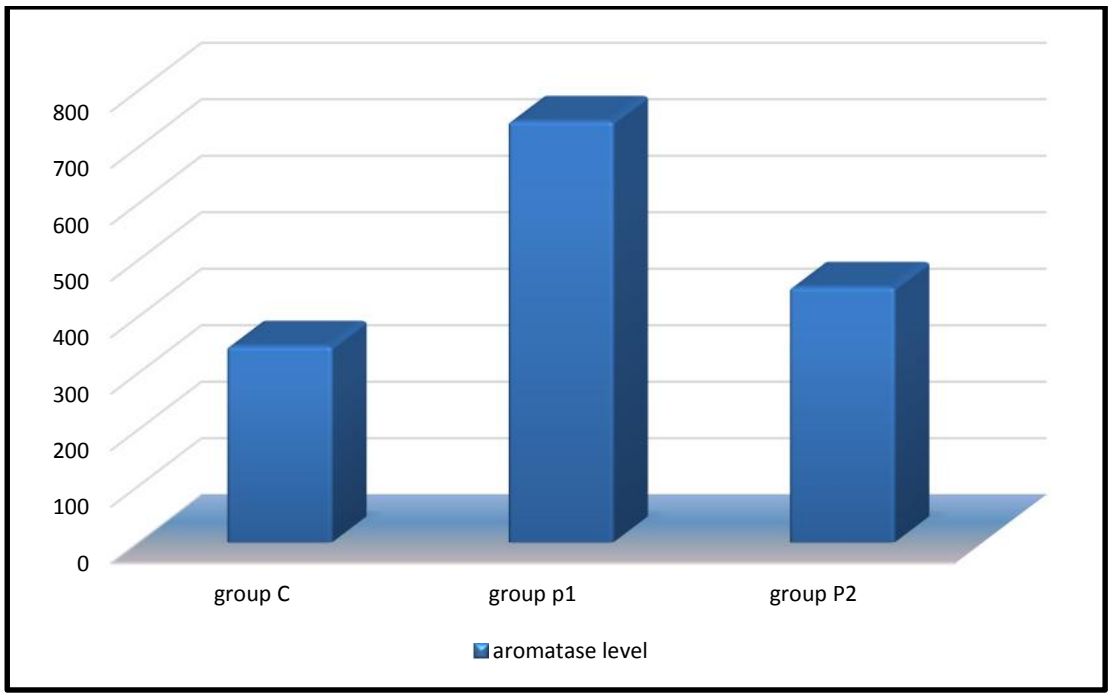



Figure (3-7): comparison of the mean aromatase between the three groups (C), (P1) and (P2).

## 3.3 Personal correlation analyses

### 3.3.1 Personal correlation analysis of aromatase

As shown in the table (3-7), serum aromatase levels of the group (P1) showed significant negative correlation with WHR while showed a significant positive correlation with weight, BMI, and estradiol (figure 3-8).

Serum aromatase levels of the group (P2) showed a negative correlation with the waist while showed a significant positive correlation with weight, BMI, and estradiol (figure 3-9).

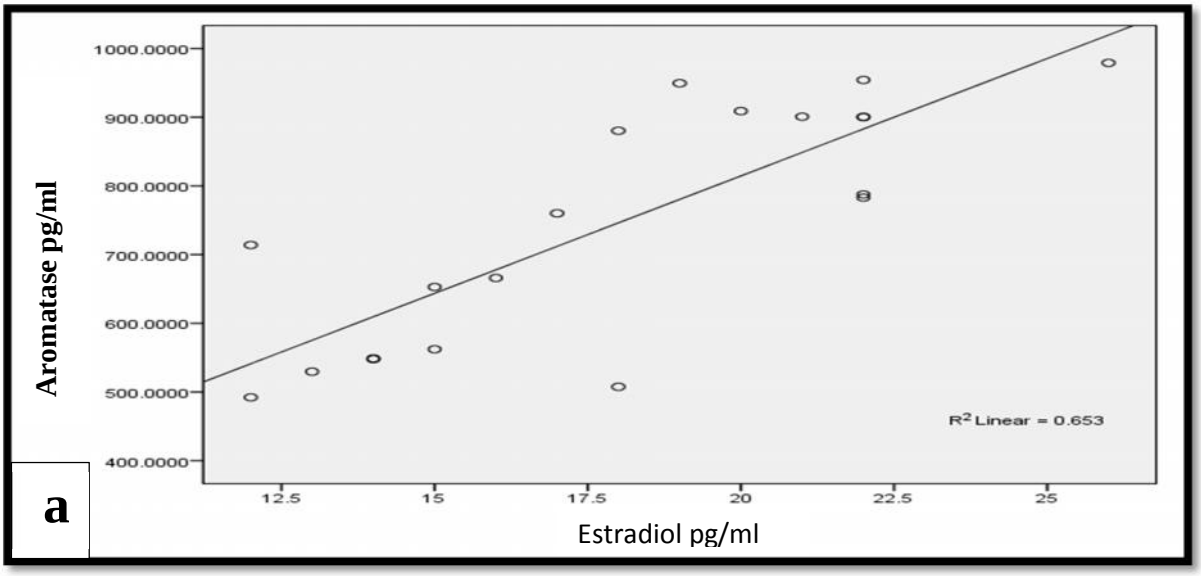
In obese postmenopausal women, aromatization of androstenedione in the adipose tissue is the major source of estrogen production and this may result in enhanced tumor growth, aromatase activity and estradiol levels have increased markedly in obese postmenopausal women due to increasing of aromatization, Key, J. [131], which that can be explaining the strong positive correlation of aromatase with estradiol and BMI in the group (P1).

Conflicting result was found in a study of Jacobs, S.[132] who found that aromatase activity did not correlate significantly with estradiol in postmenopausal patients.

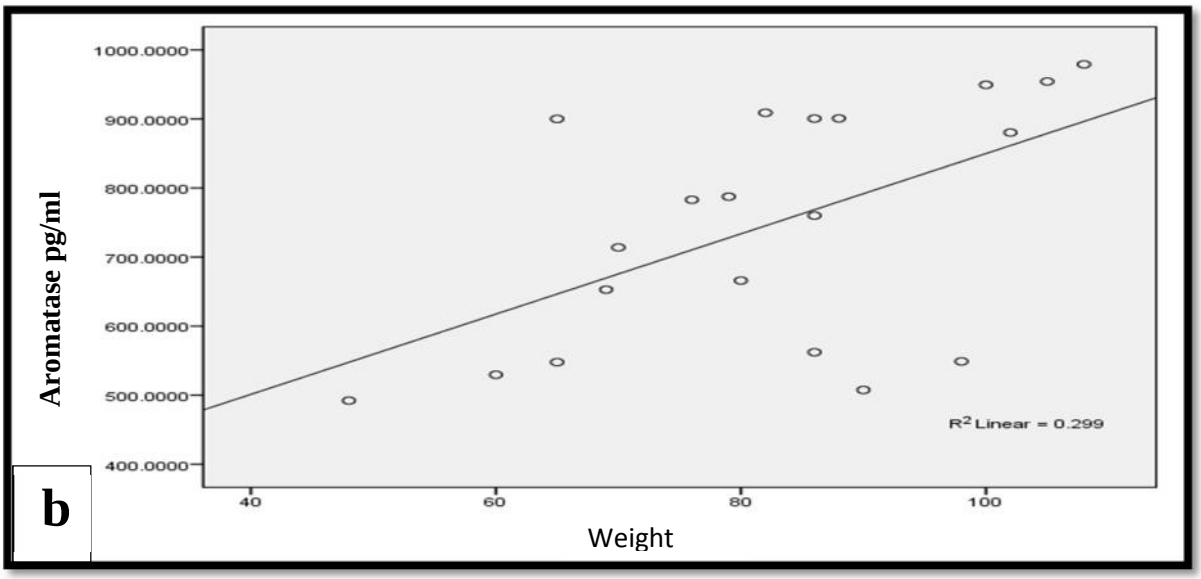
**Table (3-7):** Pearson correlation analysis of aromatase in group (P1) and group (P2) .

| Parameter | Aromatase (pg/ml) | |
|---|---|---|
| | r value group ( P1) | r value group ( P2) |
| Weight (Kg) | 0.547* | 0.427** |
| Height (cm) | -0.078 | 0.302 |
| BMI (Kg/m$^2$) | 0.62** | 0.374* |
| Waist (cm) | -0.364 | -0.161 |
| Hip (cm) | 0.228 | 0.258 |
| WHR | -0.532 * | 0.128 |
| WHtR | -0.386 | 0.215 |
| Estradiol (pg/ml) | 0.808*** | 0.573*** |

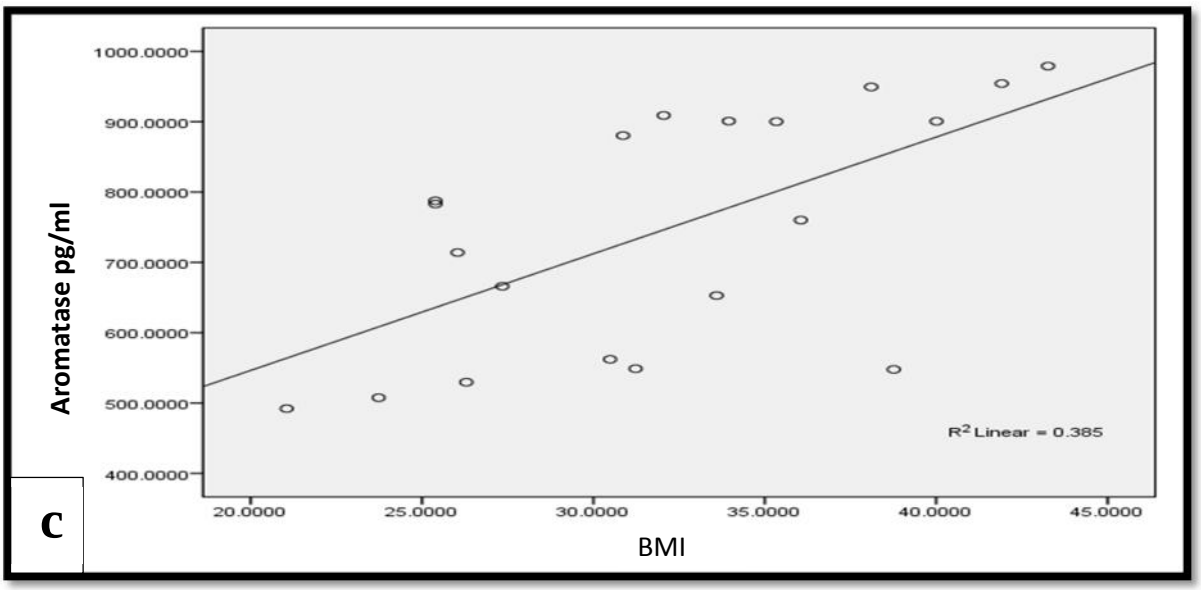
*P** ≤ 0.05; *P*<0.01; *P*<0.001; no asterisk: *P* > 0.05.


Aromatase pg/ml
1000.0000
900.0000
800.0000
700.0000
600.0000
500.0000
400.0000
12.5
15
17.5
20
22.5
25
R² Linear = 0.653
Estradiol pg/ml

**a**


Aromatase pg/ml
1000.0000
900.0000
800.0000
700.0000
600.0000
500.0000
400.0000
40
60
80
100
R² Linear = 0.299
Weight

**b**


Aromatase pg/ml
1000.0000
900.0000
800.0000
700.0000
600.0000
500.0000
400.0000
20.0000
25.0000
30.0000
35.0000
40.0000
45.0000
R² Linear = 0.385
BMI

**c**

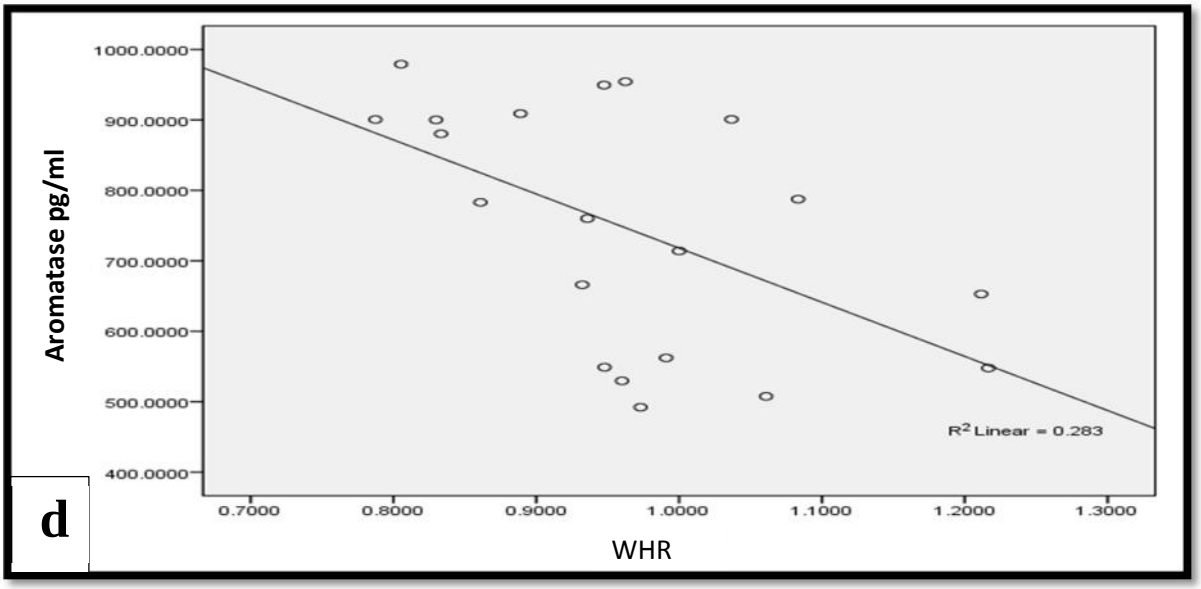
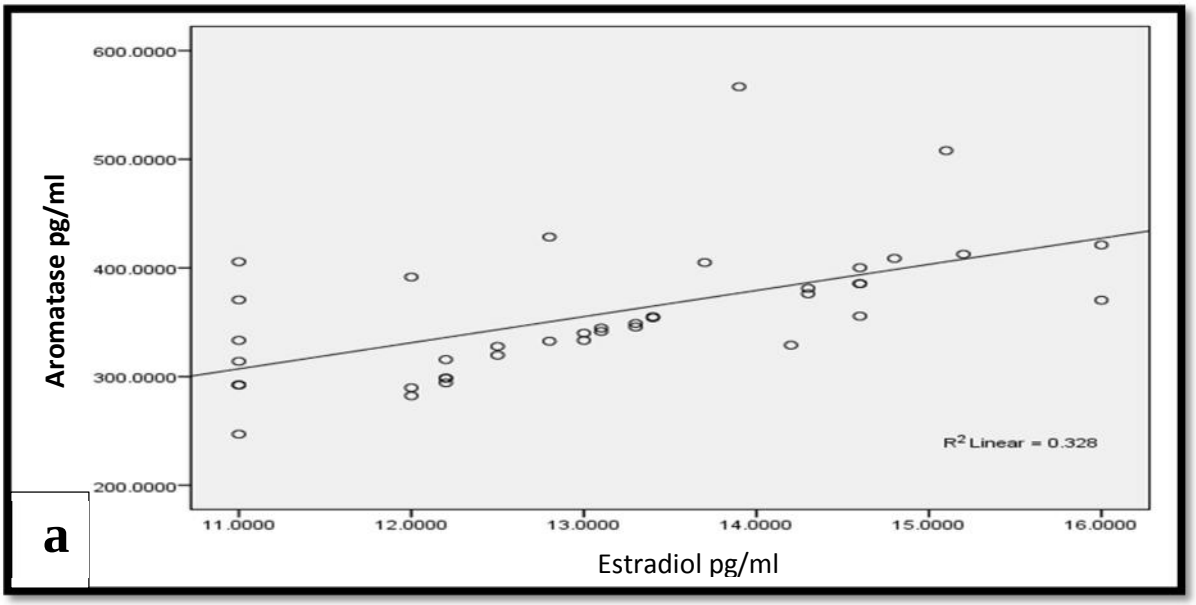



Figure (3-8): the significant correlation between serum aromatase with (a) estradiol, (b) weight, (c) BMI and (d) WHR for group (P1).



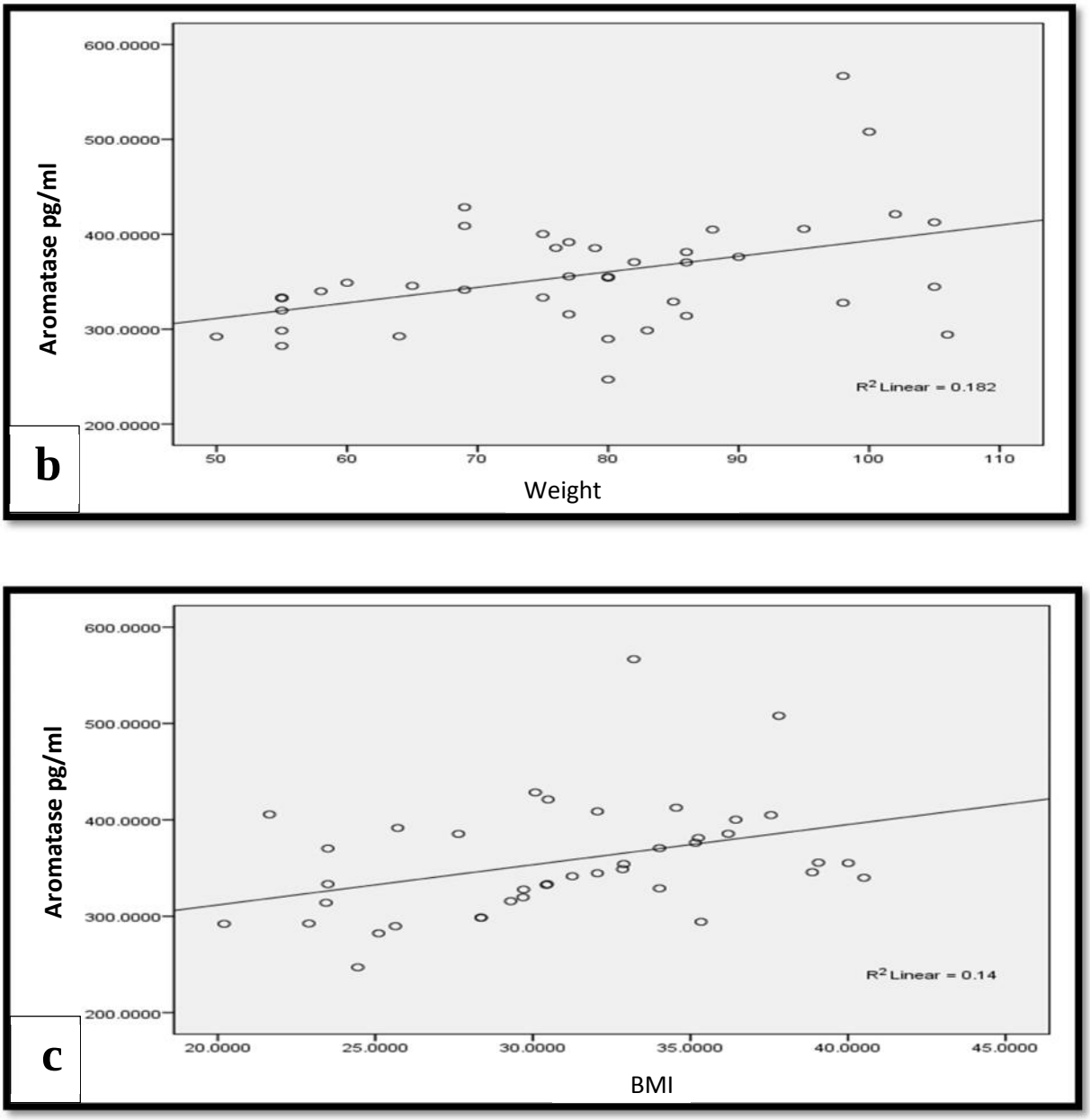



Figure (3-9): the significant correlation between serum aromatase with (a) estradiol, (b) weight and (c) BMI for group (P2).

### 3.3.2 Personal correlation analysis of estradiol

As shown in the table (3-8), serum estradiol levels of the group (P1) showed significant negative correlation with WHR while showed a significant positive correlation with weight, BMI, and aromatase (figure 3-10).

Serum aromatase levels of the group (P2) showed a negative correlation with the waist while showed a significant positive correlation with weight, BMI, and aromatase (figure 3-11).

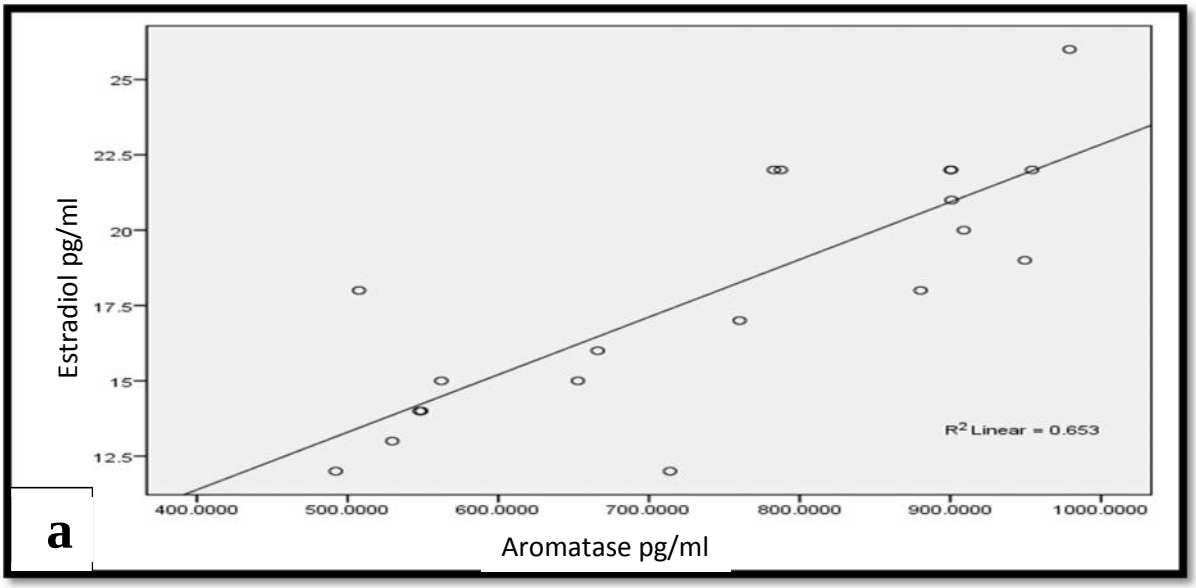
In postmenopausal women, changes in BMI are associated with changes in estradiol levels. The results suggest that fat loss by an individual can result in substantial decrease estradiol level resulting from decreasing the aromatization, Jones, M.[133]. Also, the results are in agree with Key J.[134], which were used three assay methods, showed that BMI was strongly positively correlated with the estradiol and with breast cancer risk, in all three methods.

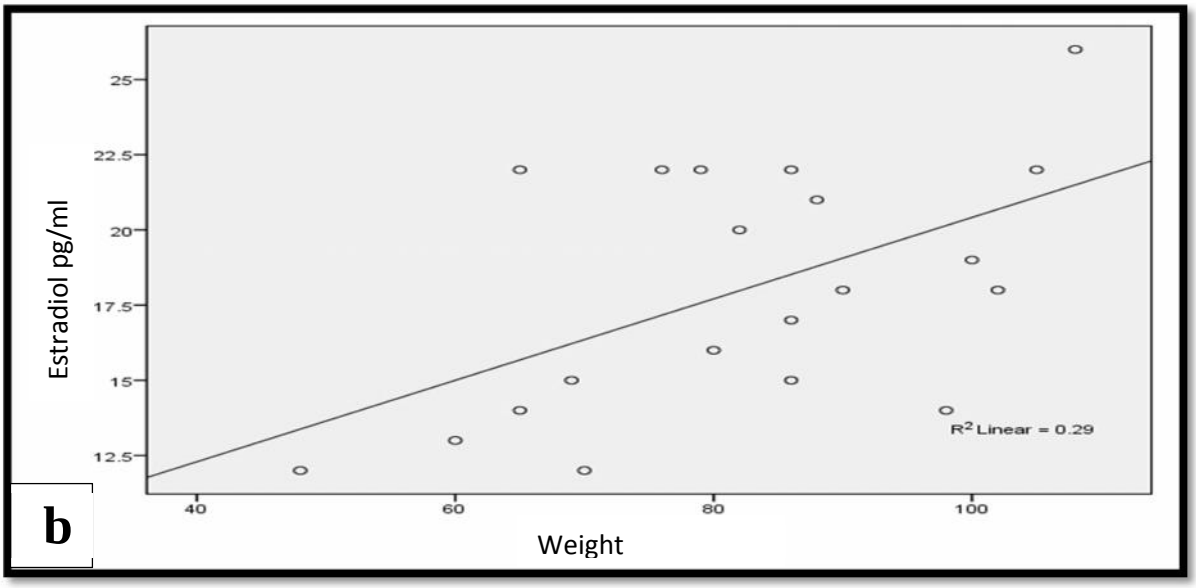
Conflicting result was found in a study of Folkert J. [135] who found that a weak positive correlation but not statistically significant was found between BMI and on-treatment estradiol levels with anastrozole.

**Table (3-8):** Pearson correlation analysis of estradiol in group (P1) and group (P2) .

| Parameter | Estradiol (pg/ml) | |
|---|---|---|
| | r-value group ( P1) | r-value group ( P2) |
| Weight (Kg) | 0.539* | 0.316* |
| Height (cm) | -0.210 | 0.215 |
| BMI ($Kg/m^2$) | 0.500* | 0.547** |
| Waist (cm) | -0.381 | -0.051 |
| Hip (cm) | 0.141 | 0.208 |
| WHR | -0.477* | 0.115 |
| WHtR | -0.42 | 0.186 |
| Aromatase (pg/ml) | 0.808*** | 0.573*** |

*P** ≤ 0.05; *P*****<0.01; *P*******<0.001; no asterisk: *P* > 0.05.


Estradiol pg/ml
25
22.5
20
17.5
15
12.5
400.0000
500.0000
600.0000
700.0000
800.0000
900.0000
1000.0000
R² Linear = 0.653
a
Aromatase pg/ml


Estradiol pg/ml
25
22.5
20
17.5
15
12.5
40
60
80
100
R² Linear = 0.29
b
Weight

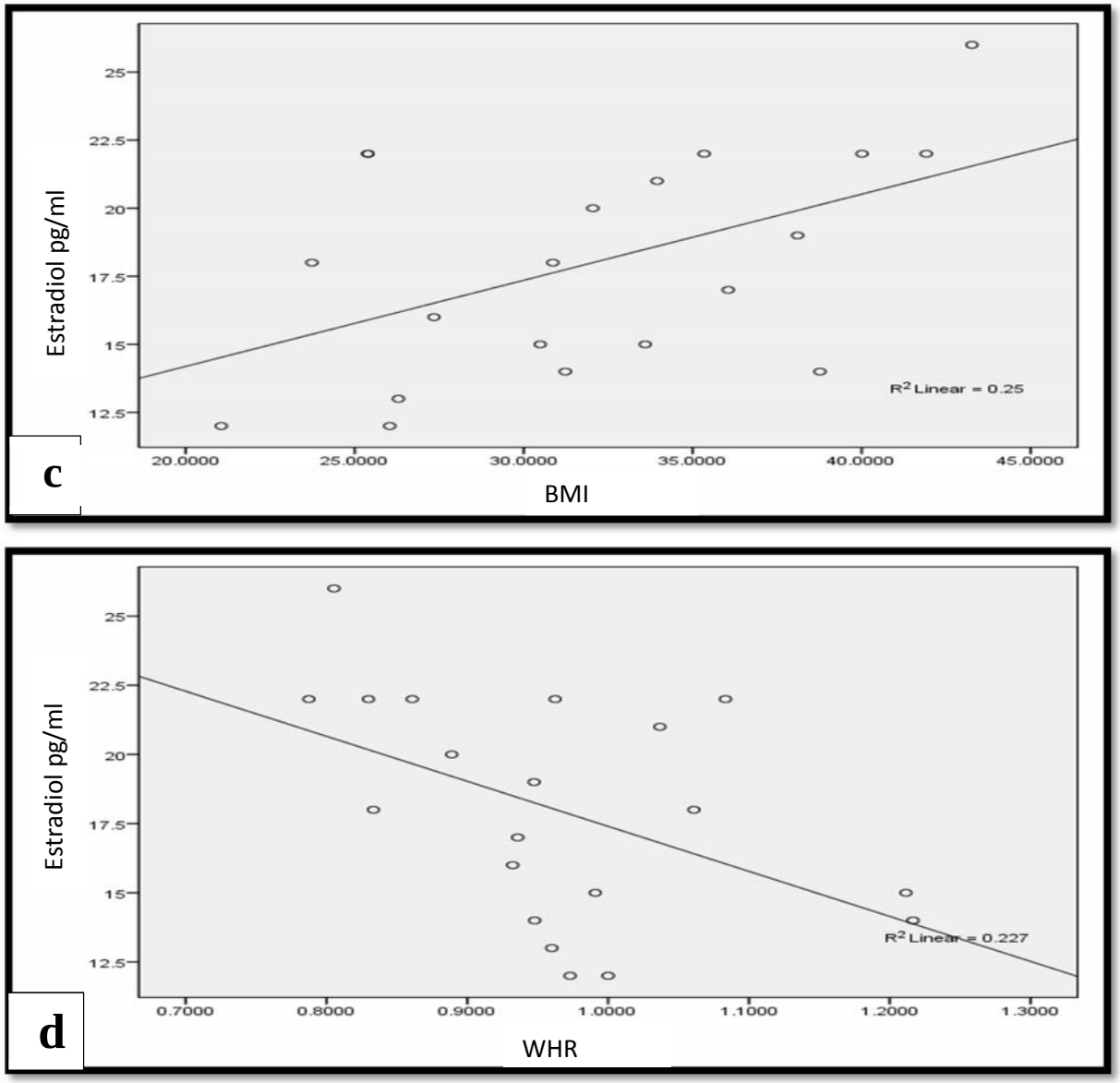
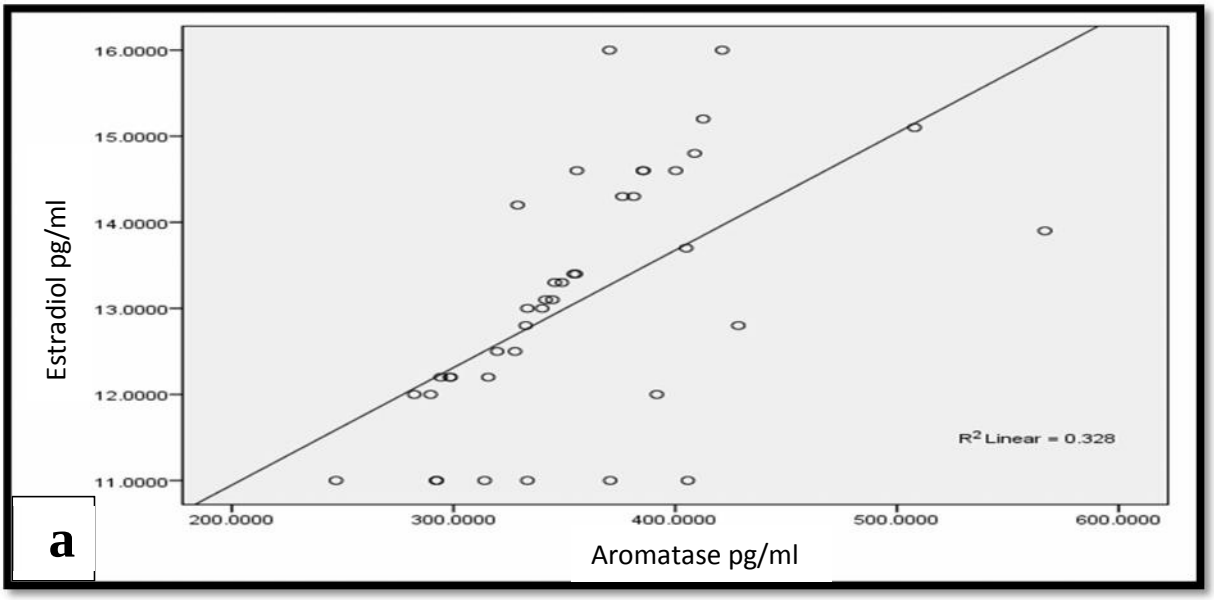
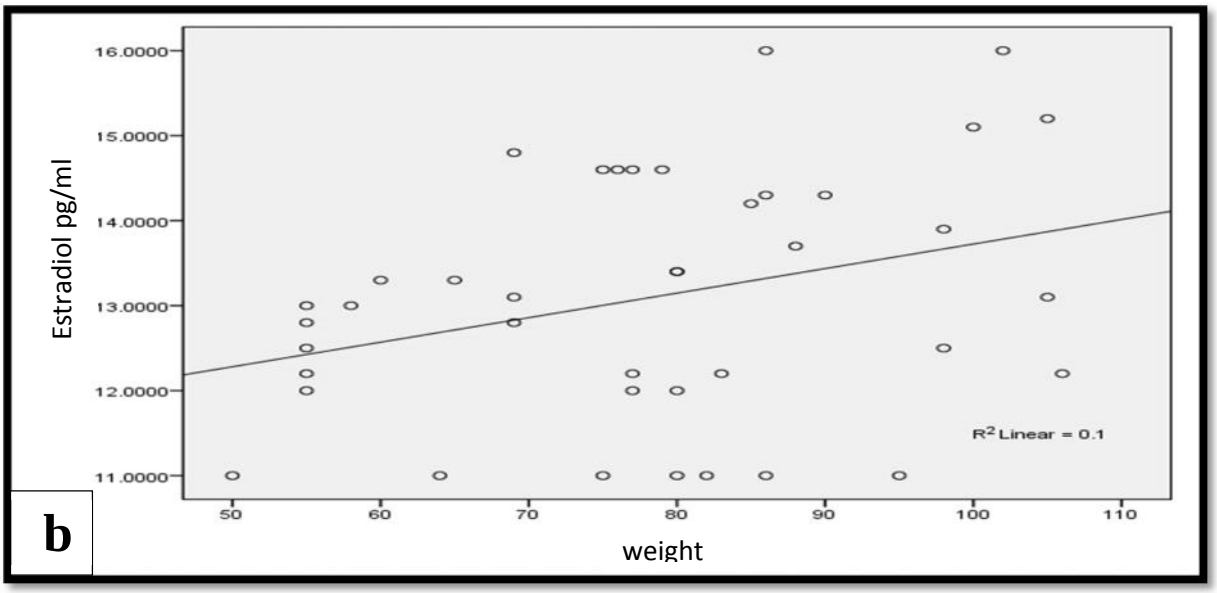



Figure (3-10): the significant correlation between serum estradiol with (a) aromatase, (b) weight, (c) BMI and (d) WHR for group (P1).


16.0000
15.0000
14.0000
13.0000
12.0000
11.0000
Estradiol pg/ml
R² Linear = 0.328
200.0000
300.0000
400.0000
500.0000
600.0000
Aromatase pg/ml
a


16.0000
15.0000
14.0000
13.0000
12.0000
11.0000
Estradiol pg/ml
R² Linear = 0.1
50
60
70
80
90
100
110
weight
b

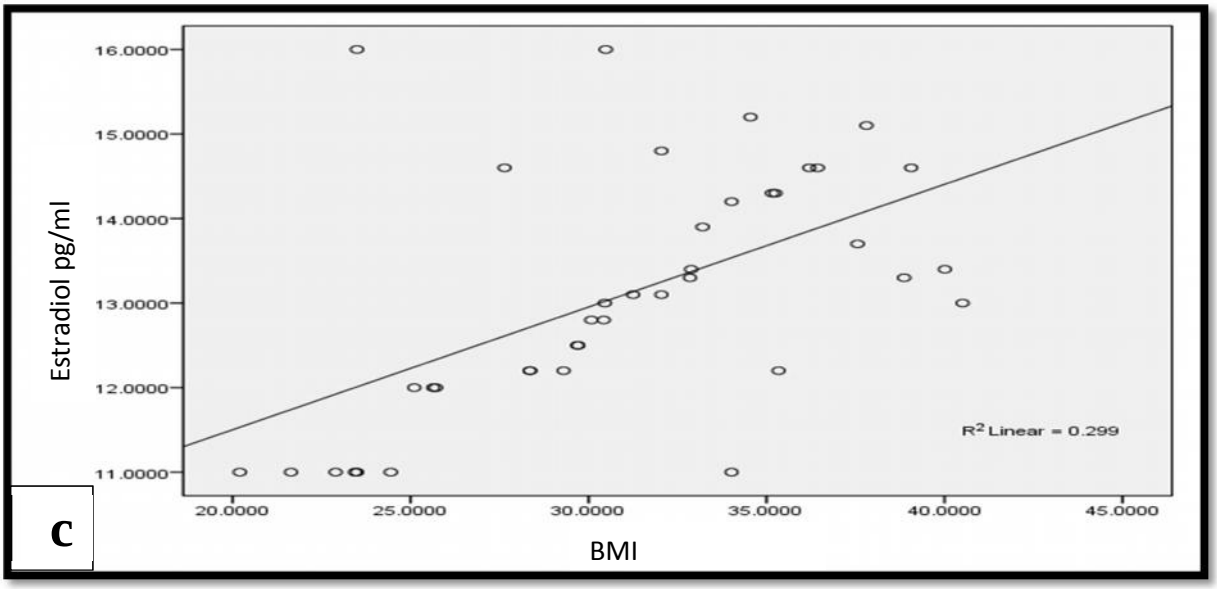



Figure (3-11): the significant correlation between serum estradiol with (a) aromatase, (b) weight and (c) BMI for group (P2).

## 3.4 Cytotoxic Effect of anastrozole on MCF7 cell line, in-vitro study (MTT assay)

The cytotoxic effect of the anastrozole was determined with three cell lines liver hepatocellular cancer cells (Hep G2), breast cancer cells (MCF-7) and Prostate cancer cells (PC-3). Analyses were done using MTT method.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cytotoxicity assay is based on the ability of a mitochondrial dehydrogenase enzymes from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark purple formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the formazan created. Results shown in Table (3-9) and Figures (3-12), (3-13) and (3-14), indicated that 400µg/ml anastrozole was the most significant cytotoxic toward all cell lines treated for 24 hours.

**Table (3-9):** Cytotoxicity effect of anastrozole (at different conc.) on HepG2, MCF-7 and PC3 tumor cell line.

| Concentration | Inhibition Rate% ±SD | | |
|---|---|---|---|
| | MCF7 | HepG2 | PC3 |
| 400µg/ml | 58.4±2.2 b | 41.7±6.9 b | 26.6±5.4 b |
| 200µg/ml | 30.9±9.8 c | 23.5±12.2 c | 15.8±3.2 c |
| 100µg/ml | 22.5±5.4 d | 17.8±2.6 cd | 10.7±1.8 c |
| 50µg/ml | 8.9±2.6 e | 10.3±2.6 d | 7.3±2.4 c |
| 25µg/ml | 7.7±3.9 e | 8.9±1.0 d | 6.3±1.7 c |
| 20µg/ml Doxorubicin | 83.97±6.1 a | 77.79±1.22 a | 70.68±4.54 a |
| LSD value | 12.483 * | 9.317 * | 9.863 * |
| * (P<0.05). | | | |

Means of different letters within the same column represented a significant difference.

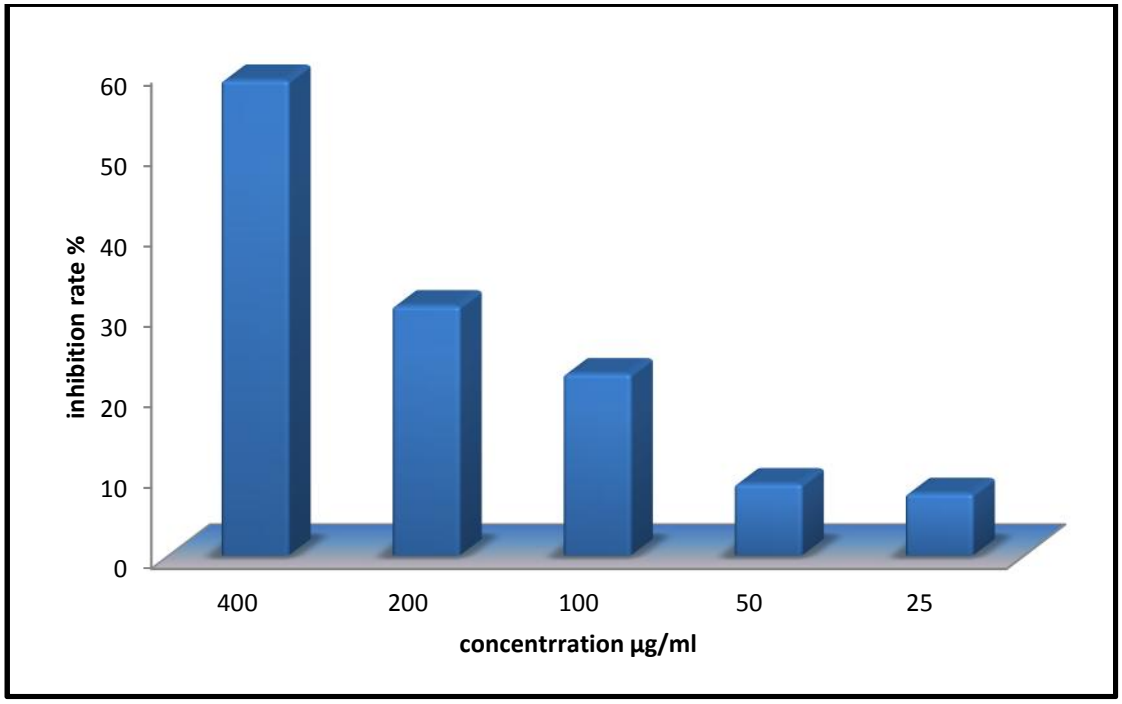



Figure (3-12): Cell inhibition rate for anastrozole on MCF-7 cell line.

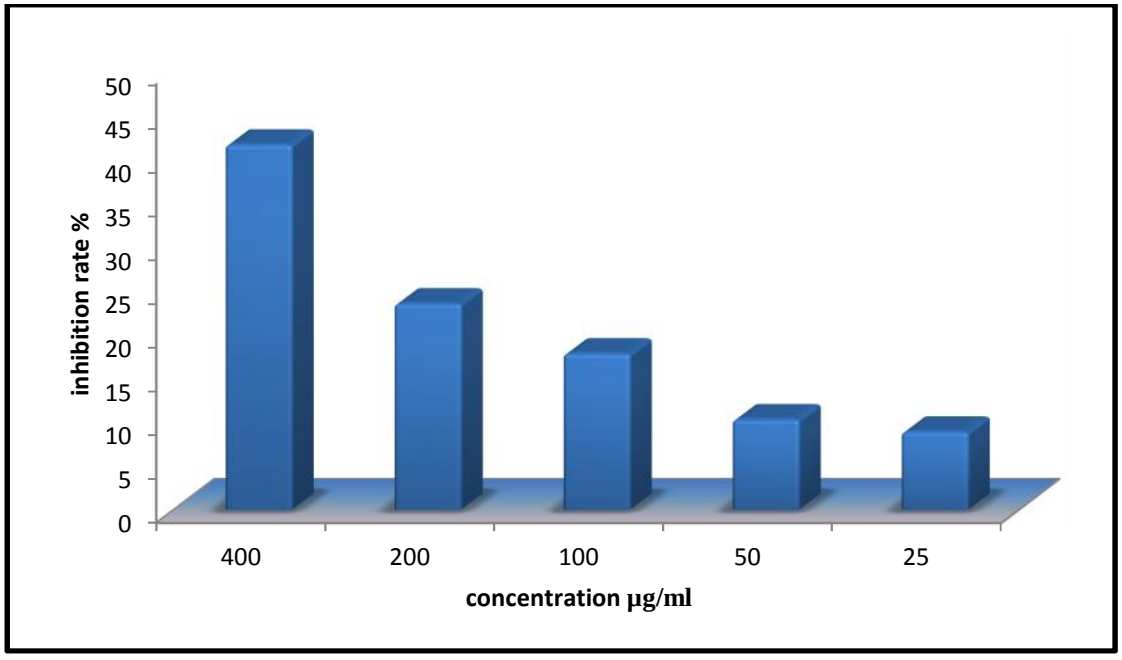



Figure (3-13): Cell inhibition rate for anastrozole on HepG2 cell line.

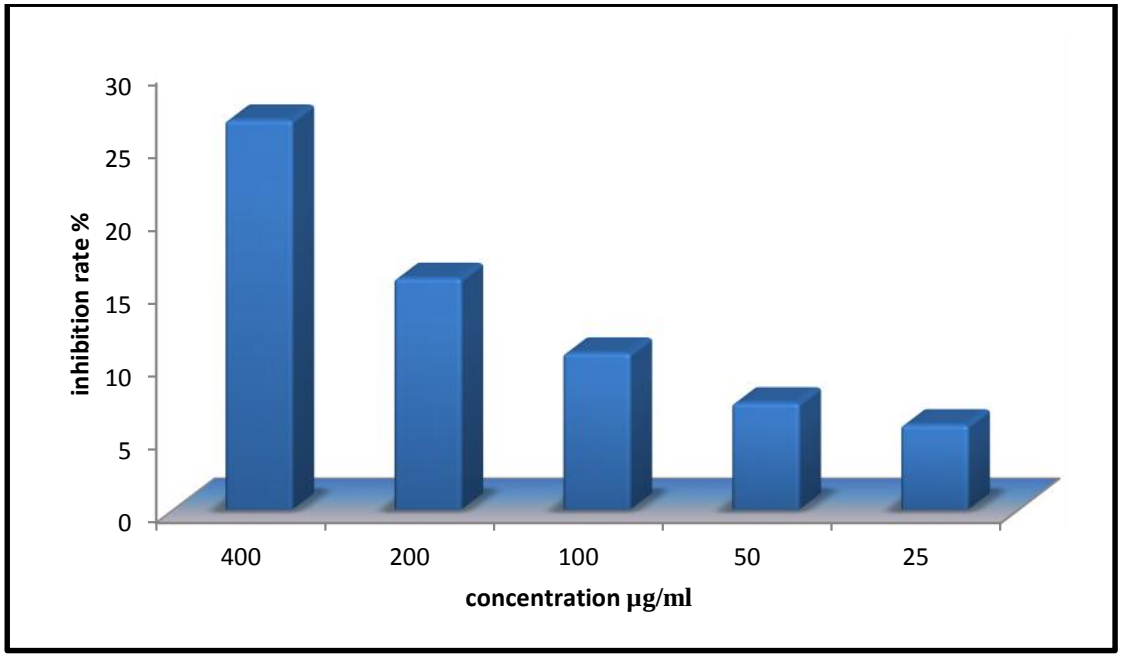



Figure (3-14): cell inhibition rate for anastrozole on PC3 cell line.

The inhibition rate values for 24 h of each dose were 83.97 for doxorubicin as standard, 58.4, 30.9, 22.5, 8.9 and 7.7 for 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml and 25µg/ml of anastrozole, respectively. The results indicated that 24 h after the administration of anastrozole to MCF7 cells, viability values were 58.4%, 30.9%, 22.5%, 8.9% and 7.7% respectively when compared to control group, which considered as 100% (Figure 3-12). The differences between standard and all experimental groups were statistically significant ($P<0.05$).

The inhibition rate values for 24 h of each dose were 77.79 for standard, 41.7, 23.5, 17.8, 10.3 and 8.9 for 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml and 25µg/ml of anastrozole, respectively. The results indicated that 24 h after the administration of anastrozole to HepG2 cells, viability values were 41.7%, 23.5%, 17.8%, 10.3% and 8.9% respectively when compared to control group, which considered as 100% (Figure 3-13). The

differences between standard and all experimental groups were statistically significant (P<0.05).

The inhibition rate values for 24 h of each dose were 70.68 for standard, 26.6, 15.8, 10.7, 7.3, and 6.3 for 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml and 25µg/ml of anastrozole, respectively. The results indicated that 24 h after the administration of anastrozole to PC3 cells, viability values were 26.6%, 15.8%, 10.7%, 7.3%, and 6.3% respectively when compared to control group, which considered as 100% (Figure 3-14). The differences between standard and all experimental groups were statistically significant (P<0.05).

The results of this study indicated that there were decreases in cell viability after treatment of three cells with aromatase inhibitor anastrozole, but the MCF-7 cells were more sensitive toward anastrozole more than other cell lines used. This was compatible with Miller R.[136] and Topcul M.[137], who suggested the mechanism of antiproliferative effects of anastrozole on breast cancer cells.

## 3.5 High content screening and analysis

The cytotoxicity of anastrozole was studied using MCF-7 cells and the thermo Scientific Cellomics Multi parameter cytotoxicity 3 Kit which enabled simultaneous measurement of six orthogonal cell- health parameters, which was cell permeability, cell count, nuclear intensity, mitochondrial membrane potential and cytochrome c level as a parameter for detecting apoptosis changes during 24 hours exposure, at various concentrations on MCF7 cell line, table (3-10).

**Table (3-10):** Effect of anastrozole on physiological parameters of MCF7 Cell Line Treated for 24 hours. MMP: is the mitochondrial membrane potential.

| **concentration** | | **Parameter test** | | | |
|---|---|---|---|---|---|
| | Cell Viability | Nuclear intensity | Cell permeability | MMP. | Cytochrome c |
| 200 µg/ml | 3022±46.3 c | 600±14.7 b | 156±6.4 b | 361±9.3 d | 535±12.7 b |
| 100 µg/ml | 3250±39.7 b | 471±9.6 c | 151±7.2 b | 426±11.8 c | 476±10.4 c |
| 50 µg/ml | 3410±42.6 a | 438±9.2 c | 141±6.9 b | 551±12.3 a | 430±10.9 d |
| 25 µg/ml | 3408±37.5 a | 436±8.6 c | 140±605 b | 505±11.3 b | 423±8.6 d |
| 0 µg/ml Doxorubicin | 1411±29.5 d | 928±11.4 a | 250± 8.6a | 245±7.4 e | 732±15.7 a |
| 0 µg/ml Untreated cell | 3411±51.6 a | 435±8.5 c | 133±6.2 b | 552± 12.3 a | 422± 9.2 d |
| LSD value | 72.471* | 135.75* | 42.69 * | 61.48* | 52.39* |
| | *(P<0.05). | | | | |

Means of different letters within the same column represented a significant difference.

High content screen analysis of anastrozole on viability, membrane permeability, mitochondrial membrane permeability and Cytochrome C realizing using MCF-7 cells was shown in figure (3-20). Screening potential drugs for toxicity is an essential aspect of the drug discovery process. In-vitro toxicity assessments performed early in drug discovery are cost- effective and fast. Cytotoxicity is a complex process affecting multiple parameters and pathways. After toxic insult, cells often undergo either apoptosis or necrosis accompanied by changes in nuclear morphology, cell permeability, and mitochondrial function, resulting in loss of mitochondrial membrane potential and release of cytochrome C from mitochondria(AL-Jailawi M.) [138]. A goal of in-vitro cytotoxicity testing is to detect the lowest dose of anastrozole that causes toxicity.

## 3.5.1 Cell Viability

The measurement of cell viability plays a fundamental role in all forms of cell culture. Sometimes it is the main purpose of the experiment, such as in toxicity assays [139]. The results of cell viability reveal that there is a significant reduction in cell viability which was 3022, 3250, 3410 and 3408 in MCF-7 cells treated with 200, 100, 50 and 25 µg/ml of anastrozole respectively (Figure 3-15). The changes in cell viability are direct positively correlated to the toxic effect of the anastrozole tested. The toxic effect on MCF7 cell viability increased with the concentration elevation and the increase percentage of cell viability were 88.6%, 95.2%, 99.97%, 99.91% and 41.46% respectively, The differences between standard and all experimental groups were statistically significant ($P<0.05$).

The most significant reduction ($P<0.05$) in cell count was at the concentration 200µg/ml of anastrozole, where it down to 3022 cells when compared to 1411 cell count at 20µg/ml doxorubicin as a standard. In this study found that MCF-7 cell viability was dose dependent after 24 hours of treatment with different concentrations of anastrozole, which affect the cellular Survival of MCF-7 cell and reduced the cell count.

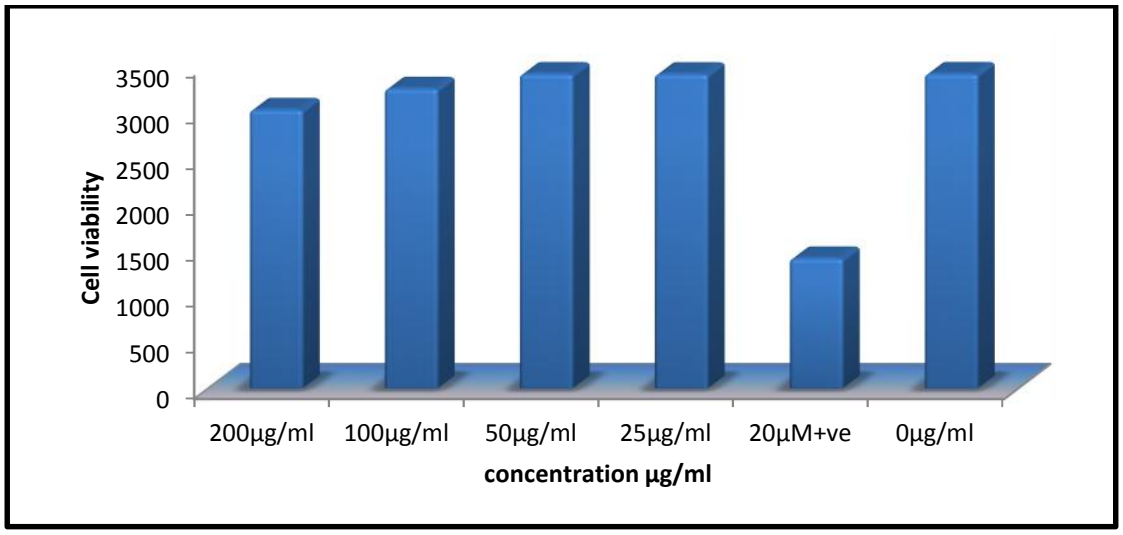



Figure (3-15): Anastrozole effect on cell viability in MCF7 cell line.

## 3.5.2 Nuclear Intensity

Nuclear condensation and fragmentation are one of the hallmarks of apoptosis [140]. This study was examined a nuclear morphological changes of MCF7 breast cancer cells that treated with anastrozole by staining the cells with Hoechst 33342 dye. The results of nuclear intensity as shown in Finger (3-16) showed that MCF-7 nuclear intensity increase significantly when treated with 200,100, 50 and 25 µg/ml of anastrozole and the increasing percentage of nuclear intensity were 38%, 8.27%, 0.7%, 0.2% and 98% respectively, the influence of anastrozole was dose dependent.

The nuclear intensity, corresponding to apoptotic changes was significantly increased to 38% after anastrozole treatment in breast cancer cells when compared with 20µg/ml standard, also with other concentrations as shown in Figure (3-16), While at concentration 100, 50 and 25 µg/ml of anastrozole wasn't found any significant difference between each other, but shown a significant when compared with 20µg/ml standard.

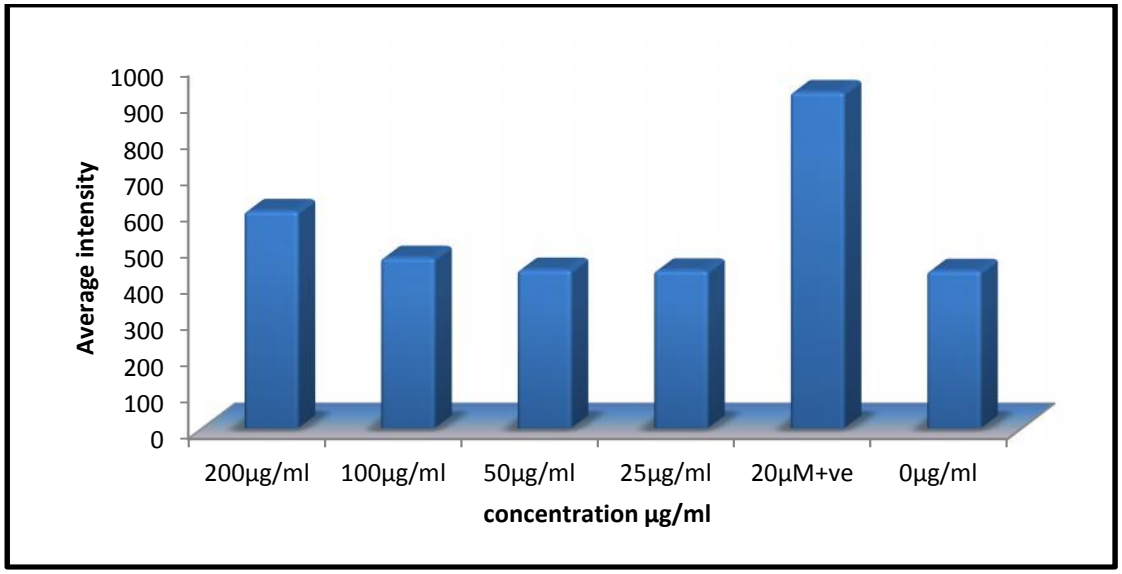



Figure (3-16): Anastrozole effect on Nuclear Intensity in MCF7 cell line.

### 3.5.3 Cell Membrane Permeability.

Dye-permeability assays are widely used in various areas of cell biology [141]. They are employed in particular to give an indication of cell membrane integrity. Because of their charged or polar nature, reporter dye molecules are unable to penetrate intact membranes but able to traverse appropriately damaged ones [142]. In this study used this as a key parameter for the evaluation of the cell compound interaction, the high doses of the anastrozole increased cell membrane blebbing leading to increasing cell membrane permeability. Figure (3-17) showed that the intensity of MCF-7 cell permeability increase significantly when treated with 200, 100, 50 and 25 µg/ml of anastrozole and the percentage of increasing intensity of the cell permeability were 17.3%, 13.5%, 6.01%, 5.26% and 87.96% respectively, these results also revealed that the effect of anastrozole was dose dependent.

The dose-dependent increased in cell membrane permeability was best significant 17.30% compared to standard at 20 µg/mL and with other concentrations, as shown in (Figure 3-17). It has been reported that changes in cell membrane permeability are often associated with a toxic or apoptotic responses, and the loss of cell membrane integrity is a common phenotypic feature of marked cytotoxicity [143].

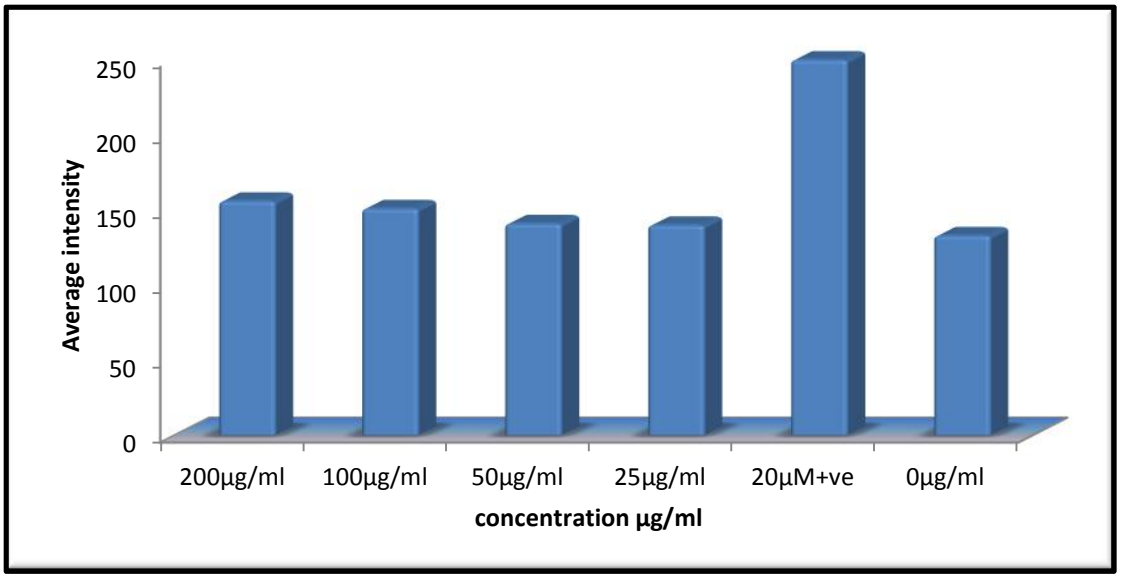



Figure (3-17): Anastrozole effect on cell membrane permeability in MCF7 cell line.

## 3.5.4 Mitochondrial membrane permeability

Membrane potential is a central feature of healthy mitochondria, and membrane depolarization is a good indicator of mitochondrial dysfunction, which is increasingly implicated in drug toxicity [144]. To better characterize the cell death signaling events in anastrozole toxicity, in this study investigated the effect of the drug on changes in mitochondrial membrane permeability.

The results from the figure (3-18) revealed that 200, 100, 50 and 25µg/ml of anastrozole caused 65.4%, 77.17%, 91.81%, 96.71% and 47% reduction in mitochondrial membrane potential intensity respectively, and the effect of anastrozole was dose dependent. The dose-dependent increased mitochondrial membrane permeability percentage was detected best significant 65.4% compared to standard at 20 µg/mL as shown in (Figure 3-18). After toxicity, cells often undergo either apoptosis or necrosis accompanied by changes in mitochondrial function, resulting in

loss of mitochondrial membrane potential and release of cytochrome c from mitochondria[145].

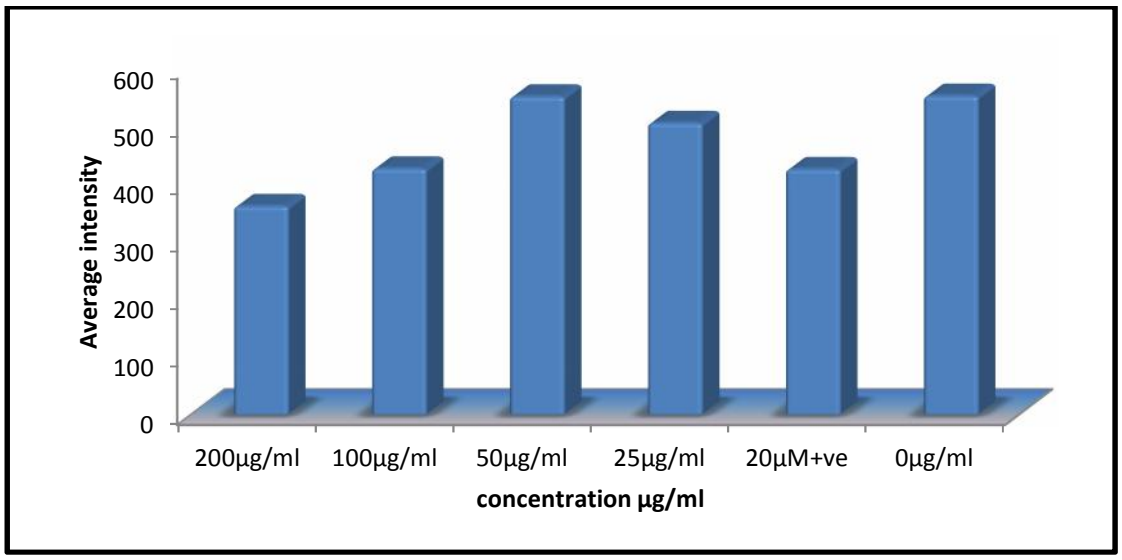



Figure (3-18): Anastrozole effect on mitochondrial membrane permeability in MCF7 cell line.

### 3.5.5 Cytochrome c

Cytochrome c plays an important role in apoptosis. The protein is located in the space between the inner and outer mitochondrial membranes. An apoptotic stimulus triggers the release of cytochrome c from the mitochondria into the cytosol [146**]**. Cytochrome c stained weakly and diffusely in control cells. In contrast, anastrozole treated-MCF7 showed strong staining around the nucleus (Figure 3-19). This suggests that treatment of breast cancer cells with compound triggered the translocation of cytochrome c from mitochondria into the cytosol. Treatment of cells with anastrozole (200, 100, 50 and 25 µg/ml) caused a significant increasing in cytochrome c releasing intensity and the increasing intensity percentage were 27.77%, 12.8% , 2.1%, 1.9% and 41.36% respectively, and the effect of anastrozole was dose dependent.

The results of this study observed that anastrozole dose-dependently induced cytochrome c significant release 27.77% compared with standard at 20 µg/ml as shown in (figure 3-19).

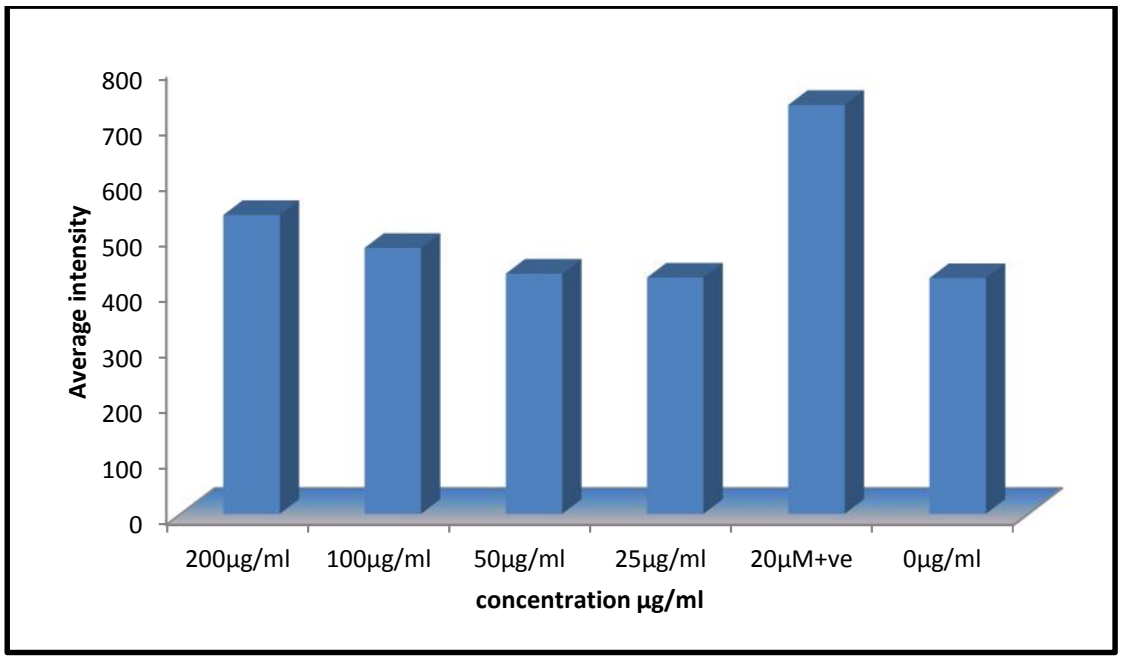



Figure (3-19): Anastrozole effect on Cytochrome C in MCF7 cell line.

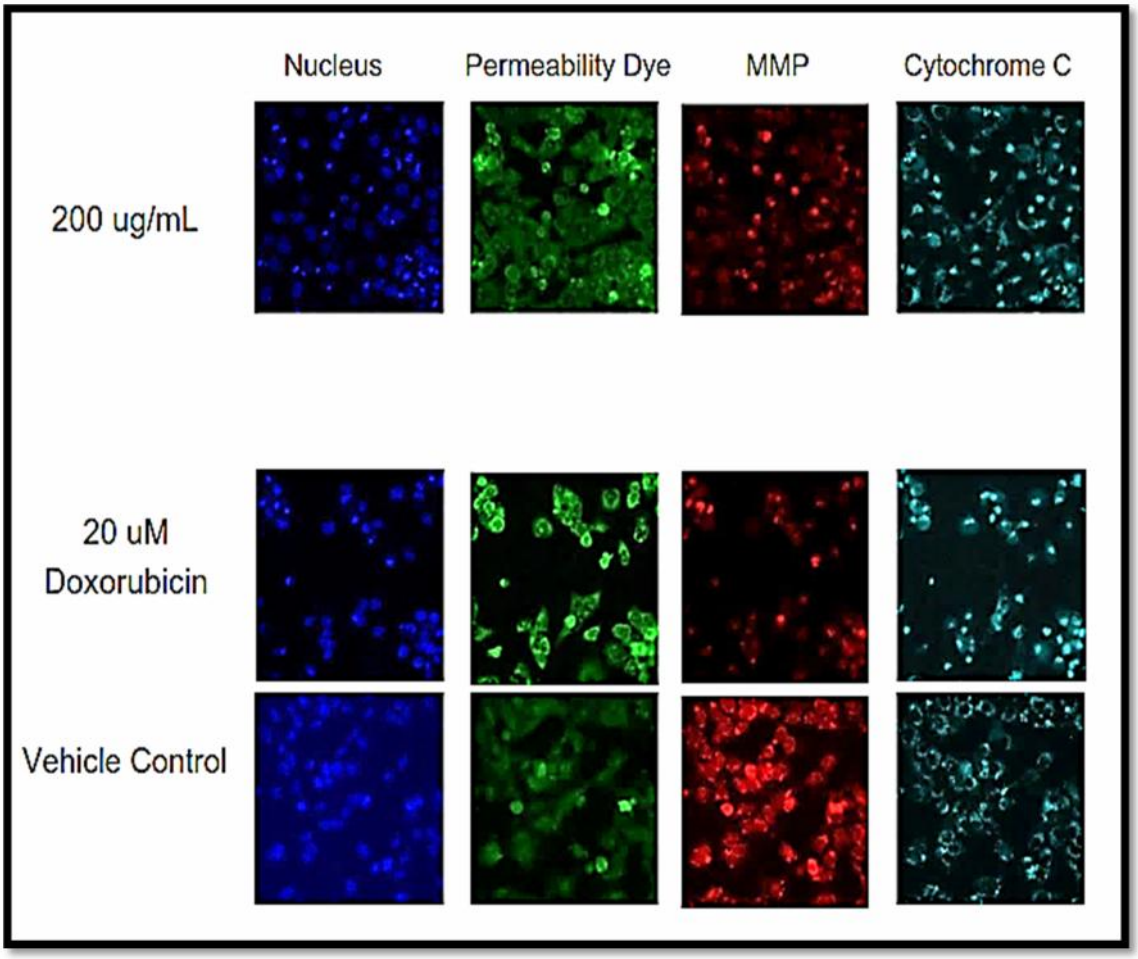



Figure (3-20): Multiparameter cytotoxicity analysis of anastrozole treated MCF7 cell line. Representative images of MCF7 cell treated with culture medium only (control), 20 µg/ml doxorubicin as positive control and more effective concentration (200 µg/ml) of anastrozole. Cells were stained with Hoechst 33342 dye (Excitation 350/Emission 461), cell membrane permeability dye (Excitation 491/Emission 509), MMP dye (Excitation552/Emission 576), and cytochrome c antibody.

# CONCLUSIONS

# AND

# RECOMMENDATIONS

# 1. Conclusions

Based on the findings of present study, it is possible to reach the following most important conclusions:

a) The results showed that increasing weight associated with increasing BMI and trend towards with increasing serum aromatase enzyme and estradiol levels, and appear positive and strong correlation between them, moreover revealed decrease significant in same parameters on patients with treatment, which that confirms the effectiveness of anastrozole and hypothesis of aromatization.

b) Anastrozole was possessing cytotoxic effect against breast cancer cells (MCF-7), liver hepatocellular cancer cells (HepG2) and prostate cancer cells (PC-3), determined in-vitro by MTT assay.

c) The HCS technique for the anastrozole showed toxic effect toward MCF7 cell line at (200µg/ml) anastrozole concentration in a dose-dependent manner with increasing in cell membrane permeability, cytochrome c, nuclear intensity, changing in mitochondrial membrane potential and decreasing in cell viability level.

d) Anastrozole has two effects on breast cancer, inhibition of aromatase enzyme that lead to inhibit aromatization mechanism and toxic effect.

## 2. Recommendation

a) According to the results, can be used aromatase analysis as monitor for detecting the response of patient to treatment.
b) Determination of aromatase activity in postmenopausal breast cancer patient treated with anastrozole depending on the duration of treatment.
c) Study the effect of anastrozole on breast cancer *in-vivo* by HCS technique.
d) Necessity of an examination of early detection of breast cancer every 6 months

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# الخلاصـــه


**مقدمة:**

يعتبر العلاج الهرموني اول علاج يستهدف السرطان بشكل مباشر ويعرف ايضا بعلاج القمع الاستروجيني، غالبا ما يستخدم بمثابة علاج مساعد للحد من خطر الاصابة بالسرطان مرة أخرى بعد الجراحة أو للحد من السرطان المنتشر. وتعتبر المثبطات الاروماتيزيه مثل (انلسترروزول) من الانواع الاكثر شيوعا في العلاج الهرموني، حيث انها تتداخل مع قدرة الجسم على إنتاج الإستروجين من الاندروجين عن طريق قمع انزيم الاروماتيز في النساء بعد انقطاع الطمث شخصوا بأنهم مصابات بسرطان الثدي ذات مستقبلات استروجين إيجابية.

تهدف هذه الدراسة الى تقييم تاثير علاج الانسترازول على اليه الارمتة وفقا لتركيز الاسترادايول وفعاليه انزيم الاروماتيز. وأيضا، لدراسة تأثيره السمي على الخلية بتطبيقه على ثلاثة أنواع من الخلايا (ثدي ، كبد و بروستات) ودراسة آلية موت الخلايا المبرمج لخلية سرطان الثدي.

**العينات، المواد وطرق العمل:**

تضمنت هذه الدراسة (80) امرأة بعد سن الياس ، تراوحت أعمارهم من (45-75) سنة. (20) عينه اصحاء تم اختيارهم كمجموعه سيطره (مجموعه C) و (60) عينة لمرضى مصابين بسرطان ثدي ذو مستقبلات استروجينيه موجبة (مجموعة P)، تم تقسيمهم الى مجموعتين وفقا لوجود او غياب العلاج : (20) مريضا دون علاج (تم تشخيصهم بالاصابه حديثا) مجموعة (P1) و (40) مريضا تمت معالجتهم بعقار الاناسترزول (1 ملغ يوميا) (مجموعة P2). في الجزء الاول من الدراسة تم تحديد مستوى كل من انزيم الاروماتيز والاسترادايول كميا للمرضى ومجموعة التحكم بواسطة فحص (ELISA). اما في الجزء الثاني من الدراسة فقد تم تحديد التأثير السمي للانسترزول على ثلاثة أنواع من الخلايا باستخدام تقنية Cytotoxic Assay والتحقيق في الية موت الخلية المبرمج للخلية الاكثر تحسسا للانسترزول باستخدام تقنية High-content screening (HCS)

## النتائج:

توصلت هذه الدراسة الى النتائج التالية:

الجزء الاول

- زيادة معنوية في معدل BMI لوحظ في مجموعة (P1) و مجموعة (P2) بالمقارنة مع مجموعة (C) ( 32.56 و 30.98 مقابل 25.44 كغم/متر$^2$)
- معدل مستوى الاستراديول المصلي اظهر زيادة معنويه في المجموعه (P1) والمجموعه (P2) بالمقارنه مع مجموعة (C) (18.10 و 13.03 مقابل 12.29 بيكوغرام/مليليتر)، بينما اظهرت نقصان معنوي في معدل المجموعة (P2)بالمقارنة مع مجموعة (P1).
- معدل سيرم مستوى انزيم الاروماتيز اظهر زيادة معنويه في المجموعه (P1) والمجموعه (P2) بالمقارنه مع مجموعة (C) (746.24 و 452.34 مقابل 347.97 بيكوغرام/مليليتر)، بينما اظهرت نقصان معنوي في معدل المجموعة (P2) بالمقارنة مع مجموعة (P1)
- ارتباط بيرسون لمستويات الاروماتيز المصلية للمجموعة (P1) اظهرت ارتباط معنوي سالب مع الخصر ،الطول و WHR بينما ارتبطت ايجابيا مع الوزن ، BMI و الاستراديول لكلا المجموعتين (P1) و(P2) .
- ارتباط بيرسون لمستويات الاستراديول المصلية للمجموعة (P1) اظهرت ارتباط معنوي سالب مع الخصر ،الطول و WHR بينما ارتبطت ايجابيا مع الوزن ، BMI و الاروماتيز لكلا المجموعتين (P1) و(P2) .

الجزء الثاني

- إظهرالتأثير السمي في ثلاث انواع من الخلايا أن اقل نسبة عيوشة معنوية للخلية عند معاملتها بالانستروزول بتركيز (400 ميكرو غرام/مليليتر) لمدة 24 ساعة ، ولكن خلايا MCF-7 كانت الاكثر تاثر بسمية المركب مقارنة بالخلايا الاخرى
- لظهر تأثير الأنلسترازول على إليه الموت المبرمج للخلية زيادة معنوية مع الزيادة في تركيز المركب على نفاذية غشاء الخلية، السيتوكروم ج و كثافة النووية عند تركيز 200ميكروغرام/مليليتر وذلك بالمقارنة مع 20 ميكروغرام/مليليتر من الدوكسوروبسين كعنصر تحكم قياسي.

- ظهر تأثير الأنلسترازول على إليه الموت المبرمج للخلية نقصان معنوية مع الزيادة في تركيز المركب على بقاء الخلية ونفاذية غشاء المايتوكوندريا عند تركيز 200ميكروغرام/ملليتر وذلك بالمقارنة مع 20 ميكروغرام/ملليتر من الدوكسوروبسين كعنصر تحكم قياسي.

## الاستنتاج:

- ظهور ارتباط معنوي موجب بين انزيم الاروماتيز والاسترادايول ،اضافة الى تسجيل انخفاض كبير في نفس المعلمات على المرضى الذين عولجوا بالانسترازول ، وهذا يؤكد فعالية الانلسترزول وفرضية ألارمتة.
- اثبت وجود تاثير سمي للانسترازول على خلايا سرطان الثدي،سرطان الكبد وسرطان البروستات باستخدام تقنية MTT.
- أظهرت تقنية HCS ان للانلسترزول تأثير سلم باتجاه خط خلية MCF7 عند (200µg/ml) بنمط يعتمد على الجرعة مع الزيادة في نفاذية غشاء الخلية، السيتوكروم ج، كثافة النووية، وتغيير في جهد غشاء الميتوكوندريا، وتناقص في مستوى قدرت الخلية على البقاء.
- وجد ان للانلسترزول تأثيرين على سرطان الثدي، الاول تثبيط انزيم الاروماتيز التي تؤدي إلى تثبيط آلية ألارمتة والثاني تأثيره السمي.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم الكيمياء

# دراسة تأثير التثبيط الاروماتيزي في مرض سوطلن الذي بعدسن اليأس ومدى تأثيره السمي على الخلايا مختبرياً

رسالة

مقدمة الى كلية العلوم /جامعة النهرين

كجزء من المتطلبات نيل درجة الماجستير في علوم الكيمياء

من قبل

**مهند وعد عبد اللطيف**

بكلوريوس 2013

بأشراف

**الاستاذ المساعد**

**د.فراس عبد حسن**

جمادي الاخر 1437

اذار 2016